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# Engenharia de tecidos aplicada à regeneração de nervo periférico

Tese de candidatura ao grau de Doutor em Ciências Veterinárias, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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**DECLARAÇÃO**

O autor desta tese declara, de acordo com o "nº 2, alínea a, do Art.º 31º do Decreto-Lei nº 230/2009" teve grande contribuição no projeto e execução do trabalho apresentado assim como na interpretação dos resultados e preparação dos manuscritos dos quais resultaram os seguintes artigos submetidos e publicados:

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# Índice

## Abreviaturas

| | |
|---|---|
| ADN | Ácido Desoxirribonucleico |
| ALCAR | Acetil-L-carnitina |
| ATP | Adenosina-trifosfato |
| BDNF | Fator de crescimento do nervo derivado do cérebro |
| bFGF | Fator de crescimento dos fibroblastos |
| cAMP | Adenosina monofosfato ácida |
| CNTF | Fator neurotrófico ciliar |
| CNTs | Nanotubos de carbono |
| DSC | Calorimetria diferencial de varrimento |
| FGF-2 | Fator de crescimento Fibroblástico |
| FK506 | Tacrólimos |
| GABA | Ácido gama-aminobutírico |
| GDNF | Fator neurotrófico derivado da linha celular da glia |
| GPC | Cromatografia de exclusão molecular |
| IGF-1 | Fator de crescimento similar à insulina-1 |
| IGFs | Fatores de crescimento similares à insulina |
| IL-8 | Interleucina 8 |
| MACs | Moléculas de adesão celular |
| MAG | Glicoproteína associada à mielina |
| MEC | Matriz extracelular do endoneuro |
| mm | Milimetros |
| ms | Milissegundos |
| MSCs | Células estaminais mesenquimatosas |
| mV | Milivolts |
| MWCNTs | Nanotubos de carbono de parede múltipla |
| NGF | Fator de Crescimento do Nervo |
| nm | Nanometro |
| NO | Monóxido de azoto |
| NT-3 | Neurotrofina 3 |
| NT4/5 | Neurotrofina 4/5 |
| OECs | Células do revestimento olfatório |

| | |
|---|---|
| p75 NTR | Recetor da neurotrofina p75 |
| PDGF | Fator beta de crescimento derivado das plaquetas |
| PPy | Polipirrol |
| PQQ | Quinona pirroloquinolona |
| PrP | Plasma rico em plaquetas |
| PVA | Poli(álcool vinílico) |
| SEM | Microscopia eletrónica de varrimento |
| SNA | Sistema Nervoso Autónomo |
| SNC | Sistema Nevoso Central |
| SNP | Sistema Nevoso Periférico |
| SWCNTs | Nanotubos de carbono de parede única |
| Tg | Temperatura vítrea |
| TGA | Análise gravimétrica |
| TGF-α | Fator de crescimento transformante alfa |
| TGF-β | Fator de crescimento transformante de tipo β |
| TrkB | Recetor da tropomiosinaquinase B |
| VEGF | Fator de crescimento do endotélio vascular |
| W / $cm^2$ | Watt/centímetro quadrado |

**Resumo**

As lesões de nervo periférico são normalmente provocadas por eventos traumáticos acidentais ou iatrogénicos, sendo que as lesões traumáticas de nervo periférico são normalmente causa de significativa morbilidade e incapacidade. As lesões de nervo periférico podem apresentar vários graus de severidade consoante o tipo de lesão a que o nervo é sujeito. Assim sendo, esta dissertação teve como objetivo geral a pesquisa de novas possibilidades terapêuticas aplicadas a lesões do nervo periférico, através de engenharia de tecidos.

No primeiro capítulo foi realizada uma revisão bibliográfica relativa à anatomofisiologia do nervo periférico, abordando a macroestrutura do sistema nervoso periférico, a estrutura macroscópica do nervo periférico, a sua histologia, assim como os processos fisiológicos envolvidos na transmissão do impulso nervos. Neste capítulo foi também brevemente revisto o tipo de lesões nervosas, os processos fisiológicos que decorrem nessas mesmas lesões e durante a regeneração nervosa assim como os fatores que influenciam a regeneração do nervo periférico. A terceira parte deste capítulo é uma súmula das diversas técnicas cirúrgicas de reparação de nervo periférico, das suas principais vantagens e desvantagens. Finalmente na quarta e última parte deste capítulo é feita uma revisão dos diferentes métodos de avaliação da regeneração e recuperação funcional do nervo periférico.

O segundo capítulo desta dissertação percorre o estado da arte relativamente à abordagem multidisciplinar no tratamento das lesões de nervo periférico e o aporte técnico e tecnológico que cada uma dessas áreas apresenta, sendo dada particular importância às armas terapêuticas que foram testadas nos trabalhos experimentais realizados no âmbito desta dissertação. Foi dada particular atenção ao desenvolvimento do biomaterial que foi testado no decorrer dos nossos trabalhos experimentais, o polímero Poli (álcool vinílico) (PVA). Este foi testado simples e ainda com diversas conjugações, associando-o a substâncias como o polipirrol, os nanotubos de carbono, o nitrato de prata ou o cloreto de magnésio, com o objetivo de aumentar condutividade deste polímero. Na mesma lógica de abordagem de possibilidades terapêuticas testadas nos trabalhos experimentais, fez-se

também uma revisão relativa às células estaminais, suas características, possibilidades de emprego terapêutico e sua compatibilidade com os biomateriais acima descritos, dando especial atenção às células estaminais mesenquimatosas.

O estudo realizado no âmbito deste Doutoramento foi planeado tendo em vista determinados objetivos específicos: a) Desenvolvimento e caracterização (físico-química e mecânica) *in vitro* de um biomaterial (PVA) e sua associação com polímeros condutores e outros iões metálicos, para obtenção de membranas e tubos-guia com alta condutividade elétrica para reconstrução cirúrgica de lesões de axonotmese e neurotmese, b) Estudo *in vivo* da aplicação de tubos-guia e membranas de PVA simples e melhorados através da associação com polipirrol e nanotubos de carbono, cuja presença aumenta a condutividade elétrica, em lesões de axonotmese e neurotmese, recorrendo ao modelo animal do nervo ciático do rato, c) Estudo *in vivo* da incorporação de sistemas celulares (MSC's) em tubos-guia de PVA e seus resultados em lesões de neurotmese recorrendo ao modelo animal do nervo ciático do rato e d) Aplicação clínica do PVA em casos clínicos de animais de companhia em medicina veterinária.

Tendo em conta os objetivos traçados, a primeira fase foi o desenvolvimento de tubos-guia do biomaterial (PVA), tornando-o passível de ser aplicado em lesões de nervo periférico, melhorando as suas qualidades físicas e mecânicas para aplicação em nervo periférico. A segunda fase foi a associação do PVA com substâncias que aumentam a condutividade elétrica, caracterização dos diferentes tubos-guia obtidos e selecionar as moléculas a associar ao PVA para os estudos *in vivo* posteriores. A terceira fase foi a aplicação dos tubos anteriormente selecionados em lesões de axonotmese e avaliação dos resultados, para posterior escolha do tubo-guia a associar ao sistema celular (MSCs isoladas da geleia de Wharton do cordão umbilical). A quarta fase foi a aplicação dos diferentes tubos-guia em lesões de neurotmese, utilizando o biomaterial selecionado em associação com MSCs.

Os estudos realizados tendo em vista o primeiro objetivo, permitiram selecionar três tipos de membranas ou tubos a testar: PVA (PVA), PVA associado ao polipirrol (PVA-PPy) e PVA associado a nanotubos de carbono (PVA-CNTs), com vista a serem aplicados em lesões de axonotmese e de

neurotmese do nervo ciático, sendo o modelo animal escolhido o rato *Sasco Sprague-Dawley*.

No primeiro trabalho *in vivo* o biomaterial (PVA, PVA-PPy e PVA-CNTs) foi aplicado em lesões de axonotmese. Após 12 semanas de avaliação foi demonstrada uma melhoria mais evidente nos grupos PVA-PPy e PVA-CNTs nos diversos testes de avaliação quer funcionais quer morfológicos.

No segundo trabalho *in vivo* foi estudada a lesão de neurotmese, onde foram aplicadas igualmente os três tipos de tubos-guia, sendo ainda testado o tubo-guia PVA-CNTs associado a células estaminais mesenquimatosas (PVA-CNTs-MSCs). Este trabalho após 20 semanas de avaliação foi demonstrada uma melhoria mais evidente no grupo PVA-CNTs-MSCs em diversos testes de avaliação quer morfológicos quer funcionais.

Finalmente, tínhamos como objetivo o teste destas soluções em casos clínicos reais, no entanto, o tempo decorrido desde o final dos testes *in vivo* e a data limite de entrega desta dissertação não permitiu a recolha e acompanhamento de casos clínicos de lesões de nervo periférico na área da clínica dos animais de companhia capaz de serem apresentados, no entanto, foi possível a aplicação das células estaminais mesenquimatosas por via intradérmica e aplicação de um penso de uma membrana de PVA em casos clínicos de lesões crónicas de pele, sem possibilidade de recuperação aplicando técnicas convencionais, apresentando resultados animadores.

Os resultados obtidos parecem-nos bastante promissores relativamente à utilização de MSCs assim como de biomateriais com elevada condutividade elétrica, sendo uma área importante a considerar em trabalhos futuros.

**Abstract**

The peripheral nerve injuries are usually caused by accidental or iatrogenic events, and are a cause of significant morbidity and incapacity in patients. The peripheral nerve injuries may present different severities depending on the type of nerve injury. Therefore, this thesis had as main objective the research of new therapeutic possibilities to be applied to nerve injuries through tissue engineering.

In chapter one a literature review regarding peripheral nerve anatomophysiology, approaching the macro-anatomy of the peripheral nerve system, the macroscopic structure of the peripheral nerve, its histology and the physiologic events involved in nerve impulse transmission. In this chapter was also reviewed the type of nerve lesions, the physiologic events arising from those lesions, events that happen during nerve regeneration and factors that influence this same regeneration. The third part of this chapter is a summary of the different surgical techniques applied to peripheral nerve repair and their main advantages and disadvantages.

The second chapter of this thesis goes through the state of the art regarding the multidisciplinary approach on the treatment of peripheral nerve lesions and the technical contribution of each area, with a special focus in the therapeutical weapons that were tested in the experimental work developed within this thesis. Special attention was given to the development and characterization of the biomaterial used in the experimental work, the polimer poli (vinyl alcohol) (PVA). This was tested pure and in combination with substances such as polypirrol, carbon nanotubes, silver nitrate or magnesium chloride, with the goal of increasing the electric conductivity of this polymer. A special focus was also given to another therapeutic weapon tested, the mesenchymal stem cells, their characteristics, therapeutic application and their compatibility with the described biomaterials.

The study within this thesis was performed considering several specific objectives: a) *In vitro* development and characterization (mechanical and physico-chemical) of a biomaterial (PVA) and its association with substances that increase the electric conductivity in order to use these membranes or tube guides to surgical correction or application in axonotmesis and neurotmesis

lesions, b) *In vivo* study of the application of membranes or guide tubes of pure PVA and PVA enhanced with polypirrol and carbon nanotubes whose presence increases electrical conductivity using the rat sciatic nerve as animal model, c) *In vivo* study of the incorporation of mesenchymal stem cells in the guide tubes in neurotmesis lesions using the rat sciatic nerve as animal model, d) clinical application of PVA in clinical cases in small animal practice.

Considering the proposed objectives, the first step was the development of PVA tube guides, making it possible to be applied in peripheral nerve lesions, improving its physical, chemical and mechanical characteristics. The second step was to associate PVA with substances that increase its electrical conductivity, characterizing the different tube guides obtained and select the tubes to be included in the posterior *in vivo* testing. Step three was the application of the pre-selected membranes in axonotmesis lesion, its results evaluation and selection of one tube to associate with MSCs isolated from the Wharton Jelly of human umbilical cord. Fourth step was application of the different tube guides in neurotmesis lesions and one group associated with MSCs.

The first studies allowed the selection of three membranes and tubes to be tested: PVA (PVA), PVA associated with Polypyrrol (PVA-PPy) and PVA associated to Carbon Nanotubes (PVA-CNTs) to be applied in axonotmesis and neurotmesis lesions in the sciatic nerve of *Sasco Sprague-Dawley* rat, defined as animal model in the experimental work.

In the first *in vivo* experimental work the chosen membranes (PVA, PVA-PPy and PVA-CNTs) were applied in axonotmesis lesions. After 12 weeks of follow-up, it was demonstrated an evident improvement in PVA-PPy and PVA-CNTs groups in several morphological and functional tests.

In the second *in vivo* experimental work the chosen tube guides (PVA, PVA-PPy and PVA-CNTs) were applied in neurotmesis lesions. Another group was added, where PVA-CNTs were associated with MSCs (PVA-CNTs-MSCs). After 20 weeks of follow-up, it was demonstrated a more evident improvement in PVA-CNTs-MSCs group in several morphological and functional tests.

The final objective proposed for this thesis involved the application of some of this therapeutic solution in real clinical cases in small animal practice. Unfortunately, the time elapsed between the end of the validation of the results

and the dead line to deliver this thesis did not allowed the collection and follow up of clinical cases of peripheral nerve lesions. However it was possible to collect cases of chronic skin injuries unresponsive to conventional treatments, where intradermal MSCs and a PVA bandage were applied, and yielding promising results.

The obtained results are very promising regarding the use of MSCs and high electric conductive biomaterials, revealing an important research area in future studies.

## Capítulo I – Revisão Bibliográfica

# Capítulo I – Revisão Bibliográfica

## 1. Anatomofisiologia do Sistema Nervoso Periférico

O estudo do nervo periférico, suas lesões, sua regeneração assim como as técnicas envolvidas na sua recuperação, obrigam a um conhecimento aprofundado do objeto de estudo, quer do ponto de vista anatomo-fisiológico quer histológico. Para melhor compreensão, parece-nos útil iniciar o estudo fazendo uma breve revisão bibliográfica sobre a histologia, anatomia e fisiologia do Sistema Nervoso Periférico (SNP).

O Sistema Nervoso permite ao organismo responder a alterações contínuas nos ambientes quer externo quer interno, controlando e integrando as atividades funcionais de órgãos e outros sistemas (Ross 2005).

Anatomicamente, o Sistema Nervoso é dividido em Sistema Nervoso Central (SNC), composto pelo cérebro e medula espinal, localizados no crânio e canal espinal, respetivamente e Sistema Nervoso Periférico (SNP) composto pelos ramos dos nervos cranianos, espinais e nervos periféricos que conduzem impulsos nervosos desde o SNC (nervos eferentes ou motores) até ao SNP (nervos aferentes ou sensoriais) (Ross 2005).

O SNP inclui todos os elementos neuronais externos ao cérebro e à medula espinal. Um **nervo periférico** é um conjunto de fibras nervosas mantidas juntas por um tecido conectivo que conduzem impulsos nervosos que se traduzem em informação sensorial ou motora entre órgãos ou tecidos do organismo e a medula espinal ou o cérebro (Ross 2005). Os nervos periféricos são os nervos cranianos e os nervos espinais. (Kierszenbaum 2002). Os corpos celulares dos nervos periféricos podem estar localizados dentro do SNC ou fora do SNC nos **gânglios periféricos**, que são basicamente e de forma simplista *clusters* de corpos celulares neuronais de onde surgem fibras nervosas que conduzem impulsos quer a partir dos gânglios periféricos quer no sentido inverso (Ross 2005).

## 1.1. Anatomia e histologia

Em 1906, e baseados nos trabalhos de Camilo Golgi e Santiago Ramón e Cajal, tornou-se consensual que o sistema nervoso era constituído por duas classes de células: **células nervosas** ou **neurónio** e **células de apoio, de suporte ou neuroglia** (Dale Purves 2001). O neurónio é a unidade funcional do sistema nervoso (Ross 2005), no entanto para o seu funcionamento são necessárias as células neurogliais que no SNC são os **astrócitos**, **oligodendrócitos**, **células microgliais** e **células ependimárias,** enquanto no SNP são as **células de Schwann** (Dale Purves 2001, Ross 2005) (Figura 1).

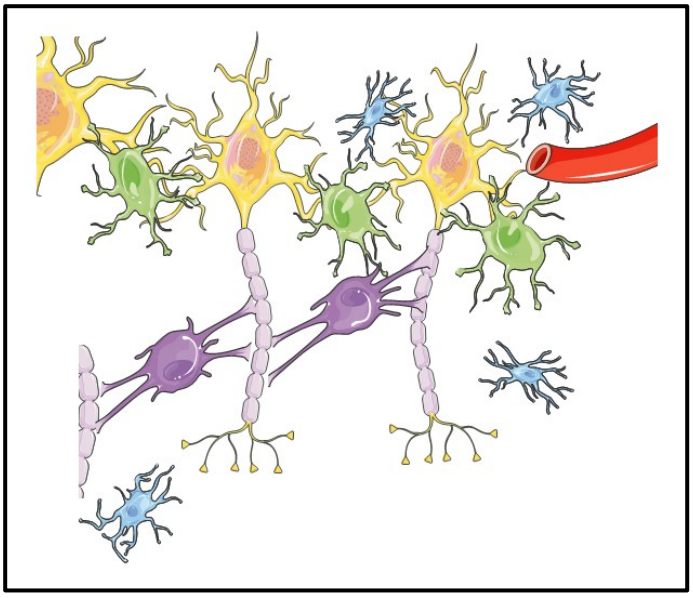

Figura 1 - Neurónios e neuroglia

No SNP, as células de Schwann são as responsáveis pelo suporte dos neurónios quer nas fibras nervosas mielinizadas quer nas não mielinizadas. Estas células são responsáveis pela produção da bainha de mielina que é uma camada rica em lípidos que circunda o axónio, isolando-o do compartimento extracelular do endoneuro, o que garante uma mais rápida condução do impulso nervoso. Nas fibras não mielinizadas, estas células também envolvem os neurónios, sendo que a sua principal função neste caso é a sua nutrição. Finalmente, as células de Schwann são também úteis na limpeza dos *debris* do SNP e conduzir a regeneração dos axónios do SNP (Ross 2005).

O termo fibra nervosa é muitas vezes empregue em diferentes formas, o que pode originar alguma confusão. Este termo pode ser conotado com o **axónio** juntamente com todas as suas coberturas (mielina e células de Schwann), ou apenas com o axónio ou ainda com qualquer processo de uma célula nervosa, seja dendrito ou axónio (Ross 2005).

As fibras nervosas individuais do SNP são envolvidas pelas células de Schwann formando fibras mielínicas e amielínicas. Nas fibras mielínicas uma célula de Schwann envolve um axónio e um axónio é envolvido por mais de uma célula de Schwann, formando um conjunto de camadas concêntricas designada bainha de mielina; nas fibras amielínicas uma célula de Schwann envolve vários axónios (Kierszenbaum 2002).

A mielina no SNC e no SNP é semelhante na sua composição proteica e lipídica. Este complexo lipoproteico é removido pelas técnicas de fixação histológicas de rotina, mas pode ser preservado quando fixada e impregnada pelo tetróxido de ósmio, que lhe confere uma coloração negra. A regulação da produção de mielina pelas células de Schwann é determinada por sinalização do axónio, estimulando as células de Schwann a sintetizar inicialmente maior quantidade de glicolípidos específicos da mielina e de seguida proteínas específicas da mielina (Mirsky R 1980, Kamholz, Awatramani et al. 1999).

O processo de formação da bainha de mielina, **mielinização**, inicia-se quando um prolongamento citoplasmático envolve o axónio e a sua membrana celular se torna polarizada em dois domínios membranares funcionalmente distintos. Quando o axónio se encontra completamente envolvido pela membrana celular da célula de Schwann, um terceiro domínio é criado, o **mesaxónio**. A bainha de mielina resulta da aposição de camadas concêntricas compactas do mesaxónio que surgem por espiralização da célula de Schwann (Kierszenbaum 2002, Lobsiger, Taylor et al. 2002, Ross 2005). Externa e contiguamente à bainha de mielina forma-se um fino colar de citoplasma perinuclear designada **bainha de Schwann** e onde se localizam a maioria dos organelos celulares. A aposição do mesaxónio da última camada sobre si mesmo encerra a espiral e produz o **mesaxónio externo** que é externamente delimitado por uma lâmina externa ou basal. O estreito espaço intercelular entre as membranas mesaxonais comunica com membrana plasmática adaxonal produzindo o **mesaxónio interno**. Quando termina o processo de espiralização, são produzidas proteínas transmembranares que criam conexões

intermembranares fazendo desaparecer o espaço entre elas, formando uma bainha de mielina compacta (Ross 2005).

A bainha de mielina de um axónio é segmentada, pois é composta por diversas células de Schwann dispostas ao longo do axónio, sendo designadas por **internódulos** e as zonas de contacto entre duas células adjacentes, desprovidos de mielina, são designados por **nódulos de Ranvier** (Kierszenbaum 2002). Estas zonas são de extrema importância para a rápida condução dos impulsos nervosos, apenas possível nas fibras mielínicas, que permitem rápidos processos de despolarização e repolarização, imprescindíveis para a génese dos potenciais de ação, possibilitando a chamada condução saltatória dos potenciais de ação (Kierszenbaum 2002, Varejão. 2003).

O nervo periférico é um feixe de fibras nervosas mantidas juntas por tecido conjuntivo de sustentação (Ross 2005). Além das células de Schwann os nervos periféricos apresentam ainda três revestimentos de tecido conjuntivo (Kierszenbaum 2002) (Figura 2):

- **Epineuro**
- **Perineuro**
- **Endoneuro**

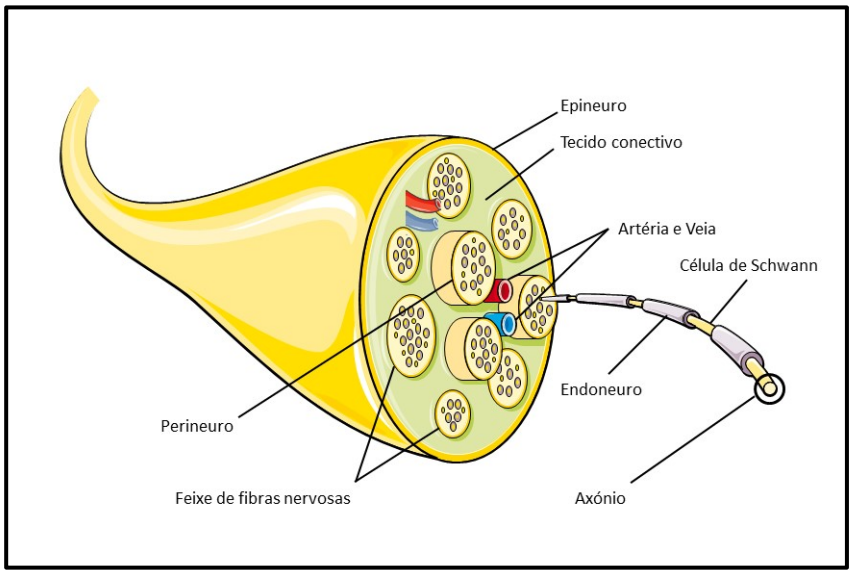


Figura 2 - Estrutura da fibra nervosa

O **epineuro** é formado por colagénio do tipo I e fibroblastos, compondo tecido conjuntivo denso e irregular que é a camada fibrosa mais externa, revestindo todo o nervo, mantendo todos os fascículos nervosos formados pelo perineuro, num feixe comum. Nos nervos de maiores dimensões, algum tecido adiposo é por vezes associado ao epineuro. Os vasos sanguíneos – ***vasa nervorum*** – presentes no epineuro, formam um plexo vascular bem desenvolvido, com numerosos vasos longitudinais, cujos ramos penetram no perineuro por onde prosseguem (Sunderland S 1985, Junqueira LC 1999, Kierszenbaum 2002, Ross 2005).

O **perineuro** é tecido conjuntivo especializado, que agrega os axónios em fascículos, fundamentalmente constituído por fibroblastos dispostos em camadas concêntricas. Esta camada apresenta duas características pouco usuais: Uma lâmina basal que circunda os fibroblastos e estes encontram-se unidos entre si por junções de oclusão – *tight-junctions* – formando deste modo uma barreira protetora – barreira hemato-neural - que evita a passagem de macromoléculas ou até de células típicas do sistema imunitário (como linfócitos ou plasmócitos) permitindo a manutenção do meio iónico das fibras nervosas contidas neste fascículo. Como atrás foi referido, este isolamento não é estanque, pois alguns ramos dos vasos epineurais penetram o perineuro, permitindo a nutrição dos neurónios inclusos nestes fascículos (Kierszenbaum 2002, Ross 2005). Uma outra função deste componente do nervo periférico é garantir a resistência elástica aos possíveis acidentes de estiramento do nervo (Sunderland S 1961), e tal é conferido por fibras de colagénio que se encontram dispostas longitudinalmente no perineuro, sendo necessário uma aplicação de uma pressão interfascicular de 750 mmHg para que ocorra uma rutura fascicular (Selander D 1978).

O **endoneuro** é a camada mais interna, constituída por tecido conjuntivo laxo, que circunda cada fibra nervosa (axónio e células de Schwann associadas) individualmente. O endoneuro é composto por alguns fibroblastos e colagénio tipo III. Alguns componentes adicionais da barreira hemato-neural, são as células endoteliais do capilares endoneurais que derivam dos *vasa nervorum* e que são delineados por células endoteliais continuas unidas por junções de oclusão (Junqueira LC 1999, Hafer-Macko CE 2002, Kierszenbaum 2002, Ross 2005). A composição do endoneuro permite assim uma maior permeabilidade do que o epineuro, permitindo que as proteínas ultrapassem esta barreira com maior

facilidade, que é fundamental para a nutrição das fibras nervosas (Mackinnon SE 2002).

Os nervos ou fibras nervosas sendo as estruturas que estabelecem a comunicação entre os centros nervosos e os órgãos sensitivos ou efetores (músculos, glândulas, etc.), que consoante o sentido em que transportam os estímulos elétricos correspondentes a informação, podem ser classificados segundo alguns autores (Junqueira LC 1999, Ghalib, Houst'ava et al. 2001) em:

- **Fibras aferentes ou nervos sensitivos** – transportam informação desde o meio ambiente e órgãos internos até aos centros nervosos e são geralmente nervos com maior densidade de fibras de menor diâmetro
- **Fibras eferentes ou nervos motores** – transportam informação dos centros nervosos até aos órgãos efetores e são geralmente nervos com menor densidade de axónios de maior diâmetro
- **Nervos mistos** – são a maioria dos nervos e são compostos pelos dois tipos de fibras descritos anteriormente

A unidade funcional do sistema nervoso, o neurónio, é uma célula altamente especializada, diferenciada e excitável. (Kierszenbaum 2002). O neurónio é composto por **corpo celular** ou **soma**, **dendritos** e **axónio**. O soma contém o núcleo celular e citoplasma perinuclear. Os dendritos são processos que surgem do soma em forma de ramos de árvore. Os ramos dendríticos são cobertos por pequenas protusões designadas espinhas dendríticas que estabelecem diversas conexões sinápticas axonais. O neurónio tem um só axónio que se origina no soma e termina numa arborização terminal designada **telodendro**, em que cada terminal apresenta uma terminação mais alargada designada **terminal sináptico** ou **botão sináptico** (Kierszenbaum 2002, Varejão. 2003, Ross 2005). Esta organização do neurónio permitiu uma especialização dos dendritos e do soma, na receção e integração de informação e do axónio na transmissão dessa mesma informação (Kierszenbaum 2002) (Figura 3).

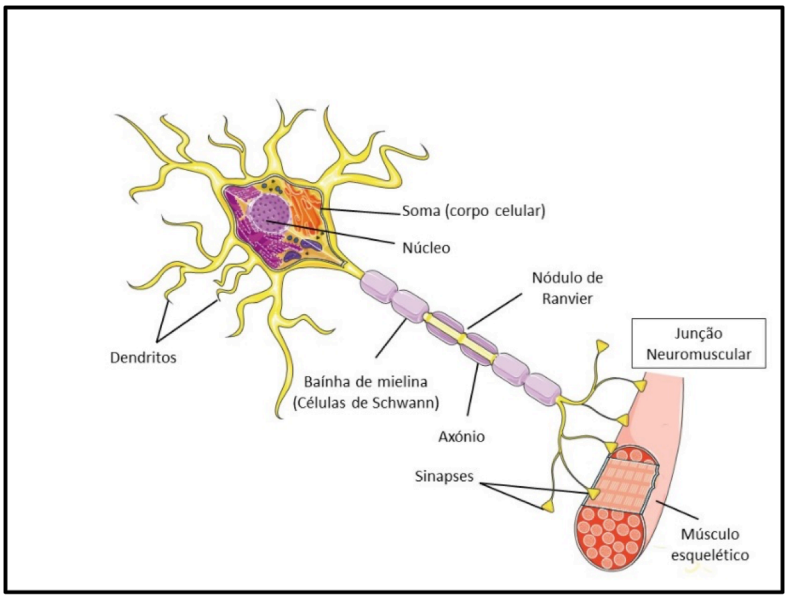


Figura 3 - Neurónio - unidade funcional do Sistema nervoso

Os neurónios podem ser classificados consoante a sua morfologia, tendo em linha de conta o número e comprimento dos prolongamentos que surgem do soma:

- **Neurónios multipolares** – apresentam muitos prolongamentos a partir do soma que apresenta uma forma poligonal. Estes neurónios apresentam um axónio e mais de um dendrito, sendo o tipo de neurónio mais abundante no sistema nervoso; São subdivididos em **Golgi tipo I e tipo II** consoante a relação entre o comprimento do axónio e da árvore dendrítica
- **Neurónios bipolares** – apresentam dois prolongamentos, um dendrito e um axónio, e são típicos do sistema visual, auditivo e vestibular
- **Neurónios pseudounipolares** – apresentam apenas um prolongamento curto (axónio), que imediatamente se divide em dois processos longos, sendo que um se dirige para a periferia e outro para o SNC (Kierszenbaum 2002, Ross 2005)

Os neurónios podem ainda ser classificados segundo a sua função (Ross 2005):

- **Neurónios sensoriais** – recebem os estímulos sensoriais provenientes dos recetores e conduzem-nos até ao SNC e incluem as **fibras somáticas aferentes** e as **fibras viscerais aferentes**

- **Neurónios motores** – conduzem os impulsos nervosos desde o SNC para os órgãos efetores, e incluem **as fibras somáticas eferentes** e as **fibras viscerais eferentes**
- **Interneurónios –** formam uma rede integrada de comunicação entre os neurónios motores e sensoriais

## 1.2. Fisiologia

A maioria dos neurónios possui apenas um axónio e um ou mais dendritos, sendo o primeiro em regra mais longo que os dendritos. As substâncias necessárias às diversas funções no axónio e nos dendritos são sintetizadas no corpo do neurónio, sendo necessários mecanismos de transporte destas substâncias para esses locais, que se designa por **transporte axonal** e é um mecanismo bidirecional. Este mecanismo bidirecional é um modo de comunicação intracelular que transporta moléculas (proteínas, neurotransmissores, lípidos) e organelos celulares ao longo de microtúbulos e filamentos intermediários entre o corpo celular e a zona terminal do axónio, e funciona em ambos os sentidos. O transporte axonal é descrito como (Kierszenbaum 2002, Ross 2005):

- **Transporte anterógrado** – é o movimento de substâncias (vesículas sinápticas, enzimas de síntese, percursores de neurotransmissores, organelos, proteínas, açucares, cálcio, aminoácidos, nucleótidos entre outros) desde o corpo celular em direção às terminações axonais
- **Transporte retrógrado** – é o movimento de substâncias desde os terminais axonais e dendríticos até ao corpo celular, transmitindo informação do estado de funcionamento do axónio (Grafstein B 1975), transportando até ao corpo celular diversas moléculas para serem recicladas no corpo celular, (fatores de crescimento, material captado por endocitose incluindo vírus e toxinas) (Kierszenbaum 2002, Ross 2005)

A condução do impulso nervoso ao longo dos axónios acontece através de um mecanismo de despolarização e repolarização da membrana celular, fazendo progredir o impulso até às sinapses onde promove a libertação dos neurotransmissores, no espaço sináptico como já foi referido anteriormente (Junqueira LC 1999). Apesar das diferenças entre os vários tipos de fibras, o

conceito básico do impulso nervoso é semelhante em todas elas e fundamenta-se naquilo que se designa por **potencial de ação**, inicialmente descrito na década de 1950 (Hodgkin and Huxley 1952, Hodgkin and Huxley 1952, Hodgkin 1964). As células nervosas quando em repouso, mantêm um potencial de membrana, ou seja, um diferencial entre a carga elétrica intracelular e extracelular, obtido pelo equilíbrio existente entre o fluxo passivo de iões de sódio e potássio, respetivamente para o interior e exterior da célula, e o mecanismo da bomba de sódio dependente da adenosina-trifosfatase (ATP) que permite a troca de 3 iões de sódio para fora da célula por troca com 2 iões de potássio para dentro da célula. Estes mecanismos permitem a manutenção de um potencial de membrana em repouso de -70 mV no interior do corpo celular. O potencial de ação representa uma inversão transitória deste potencial, que é obtido por uma alteração temporária na permeabilidade da membrana ao sódio, com uma entrada deste para o espaço intracelular através dos canais de sódio que são também estes dependentes da voltagem. Durante a despolarização, a membrana celular torna-se também mais permeável aos iões de potássio, permitindo a sua saída para o espaço extracelular, que acontece no sentido de repor o potencial de repouso, diminuindo a permeabilidade da membrana aos iões de sódio, cujos canais são inativados em 1-2 ms, efetuando assim a **repolarização da membrana celular**. Quando termina a repolarização, a membrana ainda é permeável ao potássio, permitindo a sua saída da célula promovendo uma hiperpolarização da membrana, ou seja durante um período o interior da célula é mais negativo do que em repouso, o que implica que imediatamente após ter ocorrido um potencial de ação torna-se impossível uma nova despolarização. Este momento designa-se por **período refratário**. Os mecanismos de membrana acima descritos na ausência de estímulo conduzem novamente a membrana ao potencial de repouso (Kandel, Schwartz et al. 2000, Hall and Guyton 2011).

**Sinapses** são junções especializadas entre dois neurónios ou entre um neurónio e o órgão efetor, que facilitam a transmissão do impulso nervoso do neurónio pré-sináptico para o neurónio pós-sináptico ou órgão efetor (como células musculares ou glandulares). As sinapses entre neurónios podem ser classificadas morfologicamente como (Kierszenbaum 2002, Ross 2005):

- **Axodendríticas** – ocorrem entre um axónio e um dendrito, e são as mais frequentes
- **Axosomáticas** – ocorrem entre um axónio e o corpo celular

- **Axoaxonicas** – ocorrem entre dois axónios
- **Dendrodendriticas** – ocorrem entre dendritos

As membranas pré e pós-sinápticas encontram-se separadas por um espaço designado como **fenda sináptica** (Kierszenbaum 2002, Ross 2005).

Os terminais pré-sinápticos contêm um elevado número de **vesículas sinápticas** que são transportadas para as terminações sinápticas por transporte anterógrado e possuem no seu interior mensageiros químicos – **neurotransmissores** - (acetilcolina, glutamato, ácido δ-aminobutírico – GABA, entre outros); estes são libertados por exocitose na fenda sináptica, induzido pela despolarização da membrana celular (Junqueira LC 1999, Ross 2005).

A transmissão sináptica é iniciada quando o impulso elétrico atinge o botão terminal do axónio. A condução axonal do impulso elétrico é gerada pela modificação dos canais iónicos da membrana do axónio, originando a entrada de sódio e a saída de potássio com dispêndio de ATP. A entrada de sódio é maior que a saída de potássio, pelo que ocorre uma acumulação de iões positivos no interior da célula, carregando negativamente o exterior membranar. Quando o neurónio está em repouso, a diferença de potencial entre o interior e o exterior da membrana é de -90 mV e quando a membrana se despolariza essa mesma diferença é de +35mV. A despolarização da terminação do axónio conduz a um aumento da concentração de cálcio no interior do axónio através da passagem destes iões pelos canais de cálcio sensíveis à voltagem que se reflete na exocitose das vesículas sinápticas libertando na fenda sináptica o mensageiro químico que se encontra no seu interior. Este mensageiro, por sua vez, liga-se a um recetor na membrana pós-sináptica, que pode ser adrenérgico ou colinérgico, transmitindo assim a informação. A libertação do neurotransmissor pode produzir um efeito excitatório ou inibitório da membrana pós-sináptica (Junqueira LC 1999, Ross 2005). Os neurotransmissores libertados na fenda sináptica podem ser degradados ou recapturados, sendo este último mecanismo responsável pela remoção de 80% dos neurotransmissores da fenda sináptica. Estes mecanismos são essenciais para limitar a duração do efeito de estimulação ou inibição na membrana pós-sináptica (Ross 2005).

## 2. Lesões do Sistema Nervoso Periférico

Os nervos periféricos encontram-se distribuídos por todo o organismo, pelo que lesões nervosas são relativamente frequentes e podem resultar de esmagamento, estiramento, isquemia, inflamação, seccionamento (com ou sem perda de substância) e ainda lesões iatrogénicas.

Os neurónios são células altamente especializadas que perderam no seu processo de diferenciação capacidade de sofrer mitoses, pelo que a sua morte representa uma perda definitiva. Apesar disso, os prolongamentos citoplasmáticos dos neurónios têm alguma capacidade de se regenerarem, se determinadas condições forem mantidas. Na manutenção destas condições e consequentemente na regeneração do nervo periférico, são essenciais as células da neuróglia (células de Schwann e células satélites dos gânglios nervosos), dotadas de grande capacidade de regeneração. Estes factos, levam a que após a lesão axonal, o primeiro requisito para a regeneração axonal é a sobrevivência do neurónio que é dependente de vários fatores como o tipo de neurónio, a idade ou a proximidade da lesão do corpo celular (Kierszenbaum 2002, Ross 2005).

### 2.1. Classificação das lesões

O nervo periférico está sujeito, empiricamente, a três tipos de lesão: de esmagamento ou compressão, seccionamento e seccionamento com perda de substância, por ordem crescente de gravidade. As classificações das lesões do nervo periférico mais amplamente usadas são as propostas por Seddon e Sunderland, autores com estudos de grande relevância nesta área (Seddon H 1943, Sunderland S 1951, Seddon H 1972, Sunderland S 1978, Sunderland S 1990).

A classificação de Seddon divide as lesões do nervo periférico em três categorias: Neuropraxia, Axonotmese e Neurotmese (Seddon H 1943, Seddon H 1972), por ordem crescente de gravidade.

A classificação segundo Sunderland divide as lesões em cinco categorias: Tipo I, II, III, IV e V, por ordem crescente de gravidade da lesão (Sunderland S 1951, Sunderland S 1978, Sunderland S 1985).

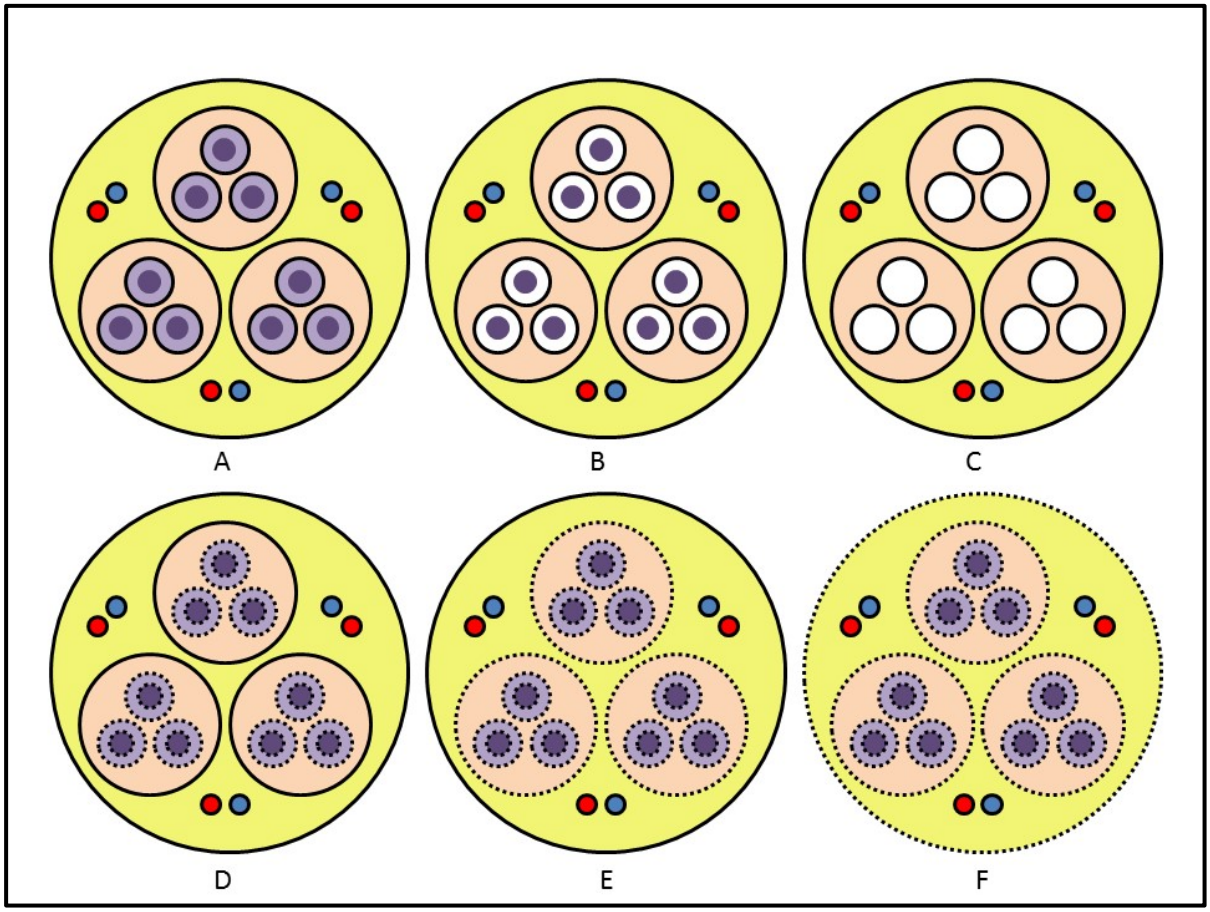


Figura 4 - A-Fibra nervosa normal; B- Lesão de Sunderland tipo I; C- Lesão de Sunderland tipo II; D- Lesão de Sunderland tipo III, E- Lesão de Sunderland tipo IV; F- Lesão de Sunderland tipo V

O termo **neuropraxia ou Lesão de Sunderland Tipo I** é a forma menos grave de lesão nervosa e define um bloqueio local na condução após compressão ou estiramento da fibra nervosa resultando na danificação da bainha de mielina com preservação da continuidade axonal, não ocorrendo assim degenerescência Walleriana, que será descrita posteriormente. Clinicamente, esta lesão manifesta-se como uma paralisia motora, sendo as fibras sensitivas e do SNA normalmente poupadas e geralmente após algumas semanas a condução nervosa no segmento afetado é geralmente completamente recuperada de forma espontânea. Os sinais clínicos resultam da maior vulnerabilidade das fibras nervosas de maior diâmetro (motores) relativamente às de menor diâmetro (sensitivos) (Seddon H 1943,

Sunderland S 1951, Seddon H 1972, Sunderland S 1978, Sunderland S 1985, Sunderland S 1990, Spiegel, Seaber et al. 1993).

A lesão de **Axonotmese ou Lesão de Sunderland Tipo II** é semelhante à anteriormente descrita, com um estádio mais avançado de compressão ou estiramento com perda da continuidade axonal e com consequente degenerescência Walleriana, mas com manutenção dos tubos do endoneuro. A recuperação funcional implica uma regeneração axonal, que geralmente ocorre de forma correta e orientada por acontecer dentro do endoneuro que foi preservado. Apesar disso, a recuperação é em regra mais morosa que no caso anterior, com prognóstico favorável e sem necessidade de cirurgia, exceto quando existe excessiva deposição de tecido fibroso no local da lesão. Os défices permanentes estão diretamente relacionados com o número de corpos celulares que possam eventualmente ter morrido, assim como danos musculares (atrofia muscular) irreversíveis que podem ocorrer enquanto o processo de reinervação não se conclui (Seddon H 1943, Sunderland S 1951, Seddon H 1972, Sunderland S 1978, Sunderland S 1985, Sunderland S 1990, Spiegel, Seaber et al. 1993).

**Neurotmese** segundo Seddon é uma lesão axonal com perda da continuidade dos axónios bem como dos seus vários revestimentos: Endoneuro (**Lesão de Sunderland tipo III**), Endoneuro e Perineuro (**Lesão de Sunderland tipo IV**), Endoneuro, Perineuro e Epineuro (**Lesão de Sunderland tipo V**). Destas lesões resulta sempre uma desorganização total da estrutura anatómica no local afetado. Neste tipo de lesão a regeneração espontânea não é possível, pelo que implica sempre intervenção cirúrgica de reparação no sentido de facilitar a orientação dos axónios do topo proximal para o topo distal o mais rapidamente possível por forma a evitar ou minimizar a formação de neuromas no local da lesão, assim como lesões irreversíveis nos órgãos alvo (como a atrofia muscular) (Seddon H 1943, Sunderland S 1951, Seddon H 1972, Sunderland S 1978, Sunderland S 1985, Sunderland S 1990, Spiegel, Seaber et al. 1993).

## 2.2. Fisiopatologia das lesões

### 2.2.1. Fisiologia da degenerescência e regeneração do Nervo Periférico

Os mecanismos envolvidos na morte do neurónio ainda não são totalmente compreendidos, mas sabe-se que surgem associadas a alterações morfológicas, processos de apoptose e de fragmentação do ácido desoxirribonucleico (ADN) (Lo, Houenou et al. 1995, Rossiter, Riopelle et al. 1996, Raff, Whitmore et al. 2002).

A perda de integridade dos axónios leva a alterações morfológicas e funcionais quer do topo proximal que continua unida ao corpo celular denominadas **reação do corpo celular** (Junqueira LC 1999) quer do topo distal designada **Degenerescência Walleriana** (Kierszenbaum 2002, Ross 2005). A lesão do axónio conduz a alterações morfológicas do soma como a perda de substância ou dissolução dos corpos de Nissl (manifestação morfológica do aumento da síntese proteica), a posição excêntrica do núcleo, o aumento das dimensões do nucléolo e edema celular, designadas no seu conjunto por **cromatólise** (Torvik 1976). As alterações do corpo celular após uma lesão axonal são proporcionais à quantidade de axoplasma perdido na lesão, podendo inclusivamente conduzir à morte celular (Ross 2005).

A sobrevivência do neurónio parece estar dependente do papel desempenhado pelo fatores neurotróficos (de fontes autócrinas ou parácrinas), produzidos pelo tecido alvo, células de Schwann, fibroblastos ou macrófagos e dos quais são exemplos o fator de crescimento do nervo (NGF), a neurotrofina 4/5 (NT4/5), o fator de crescimento do nervo derivado do cérebro (BDNF), o fator neurotrófico derivado da linha celular da glia (GDNF), diversos fatores de crescimento similares à insulina (IGFs) bem como moléculas da matriz extracelular como laminina e a fibronectina (Boyd and Gordon 2003).

A **Degenerescência Walleriana** foi descrita por Augustus Volney Waller (Waller A 1850, Stoll G 2002, Koeppen AH 2004) como o conjunto de fenómenos que se seguem a uma lesão grave do axónio, com degradação do axoplasma, destruição da bainha de mielina que é removida pelas células de Schwann e macrófagos que são mobilizados para o local de lesão. Este fenómeno é um processo intrínseco ativo do axónio associado a alguns princípios de apoptose (Beirowski B 2005), que pode ocorrer quer nos axónios do SNP como do SNC, sendo, neste último, de estabelecimento mais lento e influenciado pela idade do animal (Koeppen AH 2004).

Desde os estudos de Waller se tem investigado os fatores que influenciam a degenerescência nervosa quer no topo proximal quer no topo distal à zona de lesão, como a idade, a neuroanatomia do local, o tipo de fibras nervosas envolvidas (mielinizadas ou não mielinizadas), a espessura no axónio, o tipo de lesão, a distância da lesão ao corpo celular, o comprimento do topo distal, a temperatura, assim como o sentido em que ocorre este fenómeno (anterógrado ou retrógrado), entre outros (Chaudhry V 1992, Rodriguez JF 2004, Beirowski B 2005). Um dos principais fatores a considerar quando se pensa em regeneração de nervos periféricos é a velocidade de estabelecimento deste fenómeno. A velocidade da degenerescência Walleriana é, como foi atrás referido dependente da espessura da fibra nervosa e foi calculada, para as fibras mais espessas em cerca de 45,6 mm/dia e cerca de 252 mm/dia para as fibras mais finas (Koeppen AH 2004). Relativamente à temperatura concluiu-se que temperaturas mais altas conduzem a um aumento da velocidade deste fenómeno, tendo Merzbacher inclusivamente demonstrado a ausência de degenerescência no SNC em animais mantidos a temperaturas baixas. Este facto talvez seja devido à estimulação enzimática e depleção de fatores de crescimento que ocorre a temperaturas mais altas (Koeppen AH 2004). O tipo de lesão também interfere na direção da degenerescência, pelo que em lesões de axonotmese, esta ocorre de distal para proximal e em lesões de neurotmese ocorre no sentido inverso (Beirowski B 2005).

A degenerescência Walleriana é descrita na bibliografia como uma sequência de eventos que podem ser divididos em 5 estádios:

- **Estádio I – sobrevivência do segmento distal separado do soma**. Esta fase inicia-se imediatamente após a lesão, mas segundo alguns autores a degradação da baínha de mielina no SNP inicia-se 24 a 48 horas após a lesão (Koeppen AH 2004), dando-se também início à fragmentação do axónio. Observa-se no topo distal edema da região do endoneuro (por alteração da barreira hemato-neural) com acumulação no local de organelos celulares e mitocôndrias. No segmento proximal inicia-se um processo de degenerescência que se estende até ao nó de Ranvier mais próximo. Ao contrário do que alguns autores defendem, estudos ultra-estruturais não demonstraram que as fendas de Schimith-Lanterman são a zona mais vulnerável do axónio (Koeppen AH 2004). A duração do estádio

l varia consoante a espécie animal, o comprimento do segmento distal, a temperatura e o tipo de fibra nervosa (Chaudhry V 1992)

- **Estádio II – Desintegração do axoplasma e fragmentação axonal.** Este estádio dura poucas horas, inicia-se no local da lesão e propaga-se de forma centrífuga (degenerescência anterógrada e retrógrada) (Kierszenbaum 2002). O aspeto central deste fenómeno é a fragmentação do citoesqueleto axonal, com a desintegração granular deste e a formação de detritos amorfos, resultando no colapso das bainhas de mielina (Lubińska 1977). A calpaína é uma proteínase ativada pelo cálcio, que é a enzima responsável pela clivagem das proteínas que compõem os neurofilamentos (Varejão. 2003), pelo que numerosos estudos têm sido realizados na tentativa de inibir a sua ativação, controlando as concentrações de cálcio intracelular, condicionando desta forma esta fase da degenerescência (Glass JD 2004)
- **Estádio III – Resposta primária por parte de outros elementos celulares.** Esta fase inicia-se cerca de três dias após a lesão, onde são recrutados monócitos da corrente sanguínea que se transformam em macrófagos, macrófagos tecidulares e células de Schwann, que fagocitam os restos celulares iniciando a fase de limpeza da lesão (Griffin FW 1992, Stoll G 2002, Gordon T 2003, Avelino AM 2004, Koeppen AH 2004). Estas células permitem a preparação do espaço para se dar a regeneração subsequente, sendo que os macrófagos assumem um papel de maior preponderância devido à grande variedade de moléculas que sintetizam para a matriz extracelular: fator de crescimento dos fibroblastos (bFGF),o fator de crescimento transformante alfa (TGF-α), o fator de crescimento similar à insulina-1 (IGF-1), o fator beta de crescimento derivado das plaquetas (PDGF), o fator de crescimento do endotélio vascular (VEGF) e a interleucina 8 (IL-8). Nesta fase as células de Schwann cessam a produção de mielina e iniciam uma fase de multiplicação celular que é mais intensa em axónios mielinizados e de maior diâmetro, facto que é também induzido pela libertação de agentes indutores da mitose por parte dos macrófagos e plaquetas como PDGF, bFGF e fator de crescimento transformante de tipo β (TGF-β) (Varejão. 2003). Nesta fase ocorre também a abertura da barreira hemato-neural, cerca de 24 a 48 horas após a lesão do nervo que

parece estar relacionada com o efeito provocado pelas citoquinas assim como da histamina resultante da desgranulação dos mastócitos (Griffin FW 1992)

- **Estádio IV – resposta secundária por parte das células de Schwann e da linhagem macrofágica.** Esta fase não é temporalmente passível de ser separada de forma clara do estádio precedente. Nesta fase formam-se as bandas de Büngner, que mais não são do que proliferações de células de Schwann sob a forma de cordões orientados sobre um eixo longitudinal, delimitadas pelas lâminas basais (Cajal R 1928). Aumenta o número de macrófagos atingindo o seu máximo nos primeiros cinco dias, começando de seguida a diminuir à medida que a mielina vai sendo removida, durante o primeiro mês (Gordon T 2003). Outro acontecimento de extrema importância nesta fase é o aumento da síntese de neurotrofinas que irão promover a sobrevivência e regeneração dos neurónios (Apfel 1999, Gordon T 2003, Varejão. 2003). Finalmente, no segmento proximal, ocorre também uma proliferação das células de Schwann, que migram até ao segmento distal, permitindo o estabelecimento de contacto entre os dois topos (Cheng C 2002)
- **Estádio V – resposta final.** Esta fase ocorre após algumas semanas, sendo caracterizado por uma progressiva diminuição do número de células de Schwann assim como do seu citoplasma, desaparecimento das bandas de Büngner assim como das suas lâminas basais (Weinberg and Spencer 1978), criando um ambiente favorável ao crescimento axonal (Cheng C 2002)

A lesão neuronal conduz como vimos a uma degenerescência do axónio, mas cerca de 3 horas após a lesão, inicia-se a regeneração axonal que ocorre assim em simultâneo com a degenerescência Walleriana. O processo de cromatólise pode ser revertido, mas para tal, é necessária a sobrevivência do corpo celular (Kierszenbaum 2002).

No segmento proximal à lesão, a degenerescência geralmente ocorre até ao primeiro nó de Ranvier, criando uma zona de degenerescência Walleriana devida ao influxo de iões de cálcio e ativação das proteínases (Varejão. 2003). Nesta região podem começar a formar-se filamentos, designado brotamentos que progridem em

direção às colunas das células de Schwann mas apenas aqueles que penetram através destas podem atingir o órgão efetor (Junqueira LC 1999). O sucesso da regeneração está dependente da formação destes brotamentos, do seu número, qualidade e capacidade de alcançarem o órgão efetor (Millesi 2006). Quando o espaço entre os topos nervosos é muito grande ou quando o topo distal se perde, as fibras nervosas desenvolvem-se desordenadamente, formando uma dilatação na extremidade do axónio, designado neuroma de amputação, que é extremamente doloroso (Junqueira LC 1999).

A velocidade de crescimento dos filamentos distalmente para reinervar o órgão efetor é variável entre espécies, sendo cerca de 1-1,5 mm em humanos e cerca de 3 mm em ratos (Kierszenbaum 2002, Gordon T 2003). Mesmo em cada espécie a taxa de regeneração é também ela variável, consoante os nervos afetados, a região do nervo afetada e o tipo de lesão (Seddon H 1943); assim, a taxa de regeneração é mais baixa imediatamente após a lesão, nas lesões de neurotmese quando comparadas com as lesões de axonotmese (Seddon H 1943), no entanto a capacidade regenerativa do nervo é cerca de 66% mais baixa em lesões de axonotomia de longo termo quando comparada com lesões de transecção reparadas de imediato (Gordon T 1997). O conhecimento da taxa de regeneração é de particular importância clínica, pois permite por um lado tomar decisões relativamente à terapêutica a instaurar e por outro permite estabelecer prognósticos e monitorizar a progressão da recuperação (Luís 2008). A regeneração axonal consequente do brotamento axonal tem de ser direcionada e para que tal aconteça é essencial a proliferação das células de Schwann e para que estas proliferem é imprescindível a presença de endoneuro, pelo que na ausência destes dois elementos, a regeneração axonal é suspensa (Junqueira LC 1999, Kierszenbaum 2002). Além disto, sabe-se que os axónios são incapazes de fornecer todos os fatores neurológicos essenciais à sua própria regeneração, pelo que o alongamento axonal está dependente do material proveniente do corpo celular, que atinge o local da lesão do axónio em regeneração, por transporte anterógrado, como foi anteriormente referido (C.H. Berthold 2005).

De uma forma geral, a regeneração axonal inicia-se no segmento proximal onde surgem, nas primeiras 3 horas após a lesão, os denominados **cones de crescimento** e que são dependentes dos elementos do citoesqueleto que se encontram em trânsito no local da lesão e não do substrato que vem do corpo

celular (Snider, Zhou et al. 2002). Cajal (1928) definiu a "**zona germinativa**" como a região proximal à lesão onde ocorre o brotamento dos axónios.

Para que se inicie a remielinização, o axolema tem de entrar em contacto com as células de Schwann das bandas de Büngner e a sua extensão é determinada pelas dimensões do axónio (Hildebrand, Bowe et al. 1994).

A recuperação funcional do nervo depende não só da capacidade de os axónios atingirem o segmento distal, mas também do facto de as fibras ocuparem as colunas de células de Schwann destinadas aos locais corretos (Sunderland S 1978, Brushart T M 1988). Este facto explica por que razão as lesões de estiramento ou neuropraxia em que o alinhamento das fibras é preservado, a recuperação é total (Spiegel, Seaber et al. 1993), enquanto pelo contrário, se num nervo misto, as fibras sensitivas regeneradas se orientarem ao longo das colunas cujo destino é as placas motoras do músculo estriado e que deveriam ser ocupadas por fibras motoras, a função muscular não será recuperada (Junqueira LC 2013). No entanto, os factos descritos pela **teoria da especificidade** (Seckel BR 1986) parecem demonstrar que os axónios tendem a evitar ligações erróneas dado apresentarem um crescimento preferencial em direção aos órgãos alvo que originalmente enervavam assim como apresentarem um crescimento preferencial em direção a outro axónio em vez de outro tipo de tecido (Mackinnon SE 1985, Lundborg G 1986). Esta especificidade da reinervação depende de dois tipos de fatores (Dubový P 2004):

- **Fatores mecânicos** – correta coaptação dos dois topos nervosos e que é dependente da experiência e capacidade técnica do cirurgião; neste caso, parece existir vantagem de deixar um pequeno espaço entre os dois topos para permitir a regeneração seletiva dos axónios sensitivos e motores até ao órgão alvo ao qual estavam previamente ligados (Dubový P 2004)
- **Fatores biológicos** – correspondem aos fatores neurotróficos e neurotrópicos produzidos no local da lesão, sendo de relevar que uma grande quantidade de brotamentos axonais não atingem o segmento distal e são destruídos precocemente por falta de sinais quimiotáticos provenientes da zona distal (Brushart T M 1993, Varejão ASP 2003)

É com base nestes princípios que a produção de tubos-guia ou neurotubo, com a finalidade de orientar os brotamentos axonais e criar um microambiente no seu interior capaz de facilitar a regeneração dos nervos, tem sido cada vez mais

recomendado por diversos autores (Lundborg G 1982, Gibson KL 1991, Madison RD 1992, Hundson TW 2000, Lee WP 2000, Heijke GCM 2001, Meek M F 2002).

### 2.2.2. Fatores que influenciam a regeneração do Nervo Periférico

A seletividade, capacidade e velocidade de regeneração e crescimento das fibras motoras e sensitivas é ainda controverso (Suzuki, Ochi et al. 1998, Dubový P 2004). Embora seja consensual que as células de Schwann promovem a regeneração nervosa e que existe um sincronismo biológico que regula os diferentes sinais ambientais, é também evidente que este sincronismo ainda não está completamente compreendido (Gravvanis A I 2005). Existem no local da lesão diversas moléculas que apresentam efeitos sobre as células de Schwann e podem ser subdivididos consoante a sua origem (Chen, Yu et al. 2007): **Moléculas de Matriz Extracelular** (Laminina, Distroglicano, L-periaxina, tPA/plasminogénio, Fibrina), **Fatores Neurotróficos e Recetores** (Fator de Crescimento Derivado do Cérebro (BDNF), recetor da neurotrofina p75 (p75 NTR), Fator de crescimento Fibroblástico (FGF-2), Fator de Crescimento Transformante β (TGF-β)), **Reguladores intracelulares (**Mensageiro PI3-*Kinase/Akt*, Ciclina D1).

A **matriz extracelular do endoneuro** (MEC) é o conjunto de sustâncias que envolvem as fibras nervosas, preenchendo os espaços intrafasciculares e é limitada pelo perineuro. As células de Schwann e os fibroblastos, sob o controlo dos axónios, são as células responsáveis pela produção destas moléculas e intervêm diretamente na inibição ou estimulação do crescimento axonal (Carey JD 1983), criando um microambiente mais propício à regeneração neuronal, mas que se degrada ao longo do tempo, atrasando a regeneração axonal, deteriorando-se a um ritmo maior que o da regeneração dos motoneurónios, reduzindo ainda mais a sua capacidade de regeneração (Fu S Y 1995, Sulaiman OAR 2002). A MEC contém 4 categorias de componentes (Dubový P 2004):

- Colagénios
- Glicoproteínas não-colagénios
- Glicosaminoglicanos
- Proteoglicanos

Os fenómenos de adesão entre axónios, entre axónios e células de Schwann e entre axónios e as membranas basais, são regulados por diversas moléculas

fundamentais, genericamente designadas por **MACs** (moléculas de adesão celular) (Avelino AM 2004, Luís 2008).

A **laminina** é uma glicoproteína produzida pelas células de Schwann cujas funções biológicas são promover o crescimento e migração celular, promover a regeneração de tecidos, promover a diferenciação e adesão celular (Dubový P 2001, Akassoglou and Strickland 2002, Dubový P 2004), atuando como substrato na regeneração axonal, estimulando o crescimento axonal, tem também um importante papel na especificidade de reinervação do tecido alvo (Kauppila 1998, Dubový P 2004) e suportando um ambiente propício às células de Schwann (Chen, Yu et al. 2007). Também a **tenascina** e as **tromboplastinas** são glicoproteínas com a função de promover o crescimento axonal (Dubový P 2004). A **fibronectina** é uma glicoproteína (MAC) cujas funções são promover a adesividade entre células e destas com a membrana basal e é um promotor do crescimento axonal e de migração das células de Schwann (Tong, Hirai et al. 1994). O **ácido polissiálico** é uma molécula que é re-expressa no axónio quando este é seccionado aumentando o número de brotamentos colaterais supranumerários no local da regeneração, regulando o nível necessário de adesão axónio-axónio e mediando a degeneração dos brotamentos que se projetaram inapropriadamente nas vias cutâneas (Franz CK 2005). As **Netrinas-1 e 2** são proteínas que também parecem ter algum efeito na regeneração axonal, especialmente em lesões de neurotmese, pois os seus recetores aparecem sobre-expressados nestas lesões, mas não nas lesões de axonotmese ou em nervos normais (Madison R D 2000).

O **monóxido de azoto (NO)** é um radical livre com funções biológicas a nível do sistema nervoso central e periférico, cardiovascular e imunitário (Garthwaite J 1991, Kilbourn RG 1997, Johnson ML 1998), tendo grande importância na mediação celular da desmielinização e na indução de citotoxicidade (Conti G 2004), podendo causar morte celular por apoptose ou necrose (Bal-Price A 2000).

Os **radicais livres de oxigénio** são produtos resultantes da degradação lipídica (com forte presença nos SNC e SNP). Todos estes radicais livres, assim com o NO parecem ter um papel importante na dor neuropática e no atraso da recuperação após lesão do nervo periférico (Khalil Z 1999, Khalil Z 2001).

A **quinona pirroloquinolona (PQQ)** é uma substância com efeito antioxidante, com capacidade para estimular a regeneração do nervo periférico (Liu, Li et al. 2005).

O **ácido gama-amino butírico (GABA)** é um neurotransmissor inibitório com um papel na regulação da transmissão nociceptiva. A ação desta substância na regeneração do SNP ainda não é clara, apesar de se saber que existem alterações da sua expressão quando há lesões do nervo periférico (Polgár E 2003).

Os **fatores neurotróficos** são polipeptídeos (citoquinas e fatores de crescimento) produzidos pelo tecido alvo, pelas células da glia (no SNC), pelas células de Schwann (no SNP) e pelo neurónio (Yuen EC 2001). As principais funções destas substâncias prendem-se com o suporte do desenvolvimento, manutenção e plasticidade das diferentes populações de neurónios (Fu SY 1997, Terenghi G 1999, Chen, Yu et al. 2007), e os seus efeitos parecem estar limitados à distância entre os dois topos de cerca de 5 mm (Kuffler DP 1989, Dubový P 2004). Embora estas substâncias sejam endógenas, é possível serem adicionadas ao local da lesão, embora a sua adição de uma forma fisiológica e de acordo com as necessidades a cada momento, seja ainda difícil de controlar (Schmidt CE 2003, Chalfoun, Wirth et al. 2006). Estes fatores, assim como as proteínas da matriz extracelular, induzem o crescimento axonal por via intracelular, embora a forma como o fazem é ainda pouco clara. Até à data, é reconhecido que o resultado final ao nível da regeneração é ditado pela interferência de efeitos quer destes fatores quer das proteínas da MEC (Chen, Yu et al. 2007). Dos factores neurotróficos com influência na regeneração de nervo periférico salientam-se:

- **BDNF (Fator de crescimento derivado do cérebro)** (Sendtner M 1992, Al-Majed AA 2000a, Al-Majed AA 2000b, Boyd and Gordon 2003, Gordon T 2003, Kobayashi and Hyu 2010)
- **NGF (Fator de crescimento do nervo)** (Rich, Alexander et al. 1989, Santos, Rodrigo et al. 1998, Gordon T 2003)
- **TGF-α (Fator de necrose tumoral alfa)** e **interleucina 1α** (Wagner R 1996, Stark B 2001, Stoll G 2002)
- **CNTF (Fator neurotrófico ciliar)** (Newman, Verity et al. 1996, Siegel, Patton et al. 2000)
- **GDNF (Fator de crescimento derivado das células da Glia** (Fine, Decosterd et al. 2002)
- **Neurotrofinas 3 e 4/5 (NT-3 e NT-4/5)** (Boyd and Gordon 2003)

Algumas **citoquinas**, como a **interleucina-6** e o **fator inibitório leucémico** contribuem para a regeneração do nervo periférico (Chen, Yu et al. 2007).

A **MAG** (**glicoproteína associada à mielina**) é uma molécula que parece ter um efeito promotor da regeneração sensorial em detrimento da reinervação motora, tendo nesta um efeito inibitório (Mears S 2003, Dubový P 2004).

Finalmente, a **acetil-L-carnitina** (**ALCAR**), é uma molécula cuja atividade, previne a libertação de radicais livres de oxigénio, prevenindo assim a lesão celular, tendo uma ação neuroprotetora (Tesco, Latorraca et al. 1992, Hart AM 2004).

Também algumas hormonas parecem ter influência na Regeneração de Nervo Periférico (RNP) como as **hormonas tiroideas** que parecem apresentar um efeito positivo na regeneração de nervo periférico (Barakat-Walter I 1999). A **progesterona** que apresenta efeitos positivos, por duas vias, estimulando o crescimento e maturação axonal e accelerando diretamente o processo de mielinização (Koenig, Gong et al. 2000). A **eritropoietina** que está associada a um aumento da recuperação funcional em lesões de nervo periférico (Yin, Zhang et al. 2010)

Existem ainda alguns que são **fatores externos** ao organismo e que influenciam igualmente a regeneração do nervo como o **tipo de lesão, o período de desenervação, o local da lesão, diâmetro da fibra lesada, a idade e espécie do paciente** (Sunderland S 1985). Regra geral, é aceite que pacientes mais jovens apresentam um maior potencial de produzir brotamentos axonais em comparação com pacientes mais idosos, tendo assim uma maior capacidade regenerativa. Este facto pode ser explicado por axónios de animais jovens terem um maior número de axónios motores, ou porque a área do axoplasma dos axónios motores é maior que o dos sensitivos (Brushart T M 1988, Robinson GA 2004). É também importante referir que a interação do corpo celular e o axónio é comprometido pelo decréscimo do transporte axonal anterógrado (Brunetti M 1987) e retrógrado (Jacob J 1990), havendo um decréscimo na produção de recetores de fatores neurotróficos em neurónios mais velhos (Johnson H 1996).

O **local da lesão** parece ter grande importância quanto ao prognóstico a estabelecer. Assim tem-se verificado que quanto mais distais forem as lesões melhor é o prognóstico, o que pode ser explicado pela presença de fatores tróficos produzidos pelos órgãos alvo (Madison RD 1996, Franz CK 2005), sendo necessária

a manutenção do contacto com o órgão alvo (Brushart T M 1993, Madison RD 1996, Robinson GA 2004, Franz CK 2005, Redett R 2005, Robinson GA 2005).

São também considerados **fatores externos**, todos aqueles cuja origem é externa ao organismo e que podem ser aplicados no organismo quer por via sistémica, quer mais especificamente na zona da lesão, e que afetam a regeneração nervosa, **como campos elétricos** (Mendonça AC 2003), **campos magnéticos** (De Pedro, Pérez-Caballer et al. 2005), **ultrassons** (Sato, Motoyoshi et al. 2016), **fototerapia** (Takhtfooladi and Sharifi 2015), e outros como o **CNTF** (Siegel, Patton et al. 2000), o **ALCAR** (Hart AM 2004) ou a aplicação de **plasma rico em plaquetas – PrP** (Yu, Wang et al. 2011) e ainda a aplicação no local da lesão de **sistemas celulares** como células neurais diferenciadas *in vitro* (Luis, Rodrigues et al. 2008) ou células estaminais (Gärtner, Maurício et al. 2013, Gartner, Pereira et al. 2014). A vantagem do conhecimento destes fatores é o facto de permitir a sua manipulação tendo por objetivo o desenvolvimento de novas estratégias terapêuticas vindo ao encontro com o atual conceito de Engenharia de Tecidos.

Os **ultrassons** de baixa intensidade (0,5W/$cm^2$) parecem acelerar ligeiramente a regeneração do nervo periférico, mas quando estes são utilizados com uma intensidade de (1 W / $cm^2$) já parecem atrasar a regeneração (Lowdon, Seaber et al. 1988, Raso, Barbieri et al. 2005, Sato, Motoyoshi et al. 2016).

A **fototerapia**, com recurso a laser apresenta ainda alguns resultados contraditórios que podem ser explicados pela grande variedade e formas de aplicação deste tipo de terapêutica (Chen, Hsu et al. 2005, Takhtfooladi and Sharifi 2015). Chen e seus colaboradores (Chen, Hsu et al. 2005), apresentam resultados negativos da aplicação de laser pulsado de baixa intensidade, no entanto outros autores como Rochkind e seus colaboradores defendem que a aplicação de fototerapia laser de baixa intensidade (780 nm) apresenta efeitos positivos na regeneração nervosa (Anders, Geuna et al. 2004, Rochkind, Leider-Trejo et al. 2007, Rochkind, Geuna et al. 2009).

A **estimulação elétrica** parece melhorar a regeneração nervosa, através da sincronização da reinervação do topo distal, embora alguns autores defendam que não parece apresentar qualquer melhoria na velocidade de regeneração (Al-Majed AA 2000a, Al-Majed AA 2000b, Brushart TM 2002, Brushart T M 2005) no entanto estudos mais recentes defendem o contrário (Ashour, Elbaz et al. 2015). Willand, conclui que a estimulação elétrica de baixa intensidade, aplicada diretamente,

melhora a regeneração morfológica e funcional em lesões de axonotmese, provavelmente por atrasar a degeneração axonal e estimular a formação de brotamentos – fenómeno designado na bibliografia por "*sprouting"* e por acelerar a regeneração da bainha de mielina (Mendonça AC 2003, Willand, Nguyen et al. 2015). Outros mecanismos que explicam a melhoria da regeneração nervosa através da estimulação elétrica são: i) o recrutamento de mais motoneurónios em regeneração que penetram mais cedo no topo distal (Brushart TM 2002), ii) e a estimulação do corpo celular aumentando a produção de BDNF e TrkB (Al-Majed AA 2000a, Al-Majed AA 2000b), que por sua vez interfere com a regulação do cálcio e da Adenosina monofosfato ácida (cAMP) (Brushart TM 2002), evitando a entrada de iões de $Ca^{2+}$ e $Na^{+}$ para o interior da fibra e a saída de $K^{+}$ para o seu exterior, evitando que o axoplasma se torne positivo ao mesmo tempo que no interior do axónio estimula o transporte axonal (Mendonça AC 2003). Al-Majed demonstrou que 1 hora de estimulação elétrica originou um aumento de 35% no número de neurónios originais aferentes que reinervam o músculo alvo correto (Al-Majed AA 2000b), enquanto Brushart demonstrou que a aplicação de uma hora de estimulação elétrica no momento da cirurgia, promove o retorno dos axónios sensoriais ao seu tecido de origem (Brushart T M 2005). Vários autores defendem que correntes elétricas de baixa intensidade apresentam mais vantagens e efeitos mais positivos na regeneração do nervo periférico (Mendonça AC 2003).

Alguns autores defendem que o ***stress*** parece inibir a regeneração do nervo periférico, e apesar de este facto ser ainda controverso e pouco claro, são avançadas como explicações as seguintes hipóteses (Ohara, Kawamura et al. 1991, Amako and Nemoto 1998):

- Efeito da hormona corticotrófica na regeneração do nervo periférico
- Reações e reflexos anormais via sistema nervoso autónomo
- Influência do *stress* no sistema nervoso central provocando a libertação de agentes neuroquímicos endógenos capazes de afetar a regeneração neuronal
- Influência direta do *stress* na formação de tecido conjuntivo e formação de tecido cicatricial
- Alterações nutricionais que levem à inibição da síntese de matriz pelo nervo ou transporte axonal

- Alterações endócrinas ou do Sistema nervoso Autónomo (SNA) que afetem o sistema vascular do nervo ou barreira hemática

Outros agentes como alguns fármacos imunossupressores, o **FK506** (tacrólimos) e a **ciclosporina A**, parecem ter efeitos benéficos na regeneração do nervo periférico (Gold BG 1995, Hebebrand D 1997, Sulaiman OAR 2002, Gordon T 2003, Udina, Voda et al. 2003, Udina E 2004, Brenner MJ 2005, Sosa I 2005), no entanto e mais recentemente num estudo clínico de fase II em humanos, o FK506, apesar de se ter revelado seguro, não demonstrou nenhum efeito benéfico na regeneração de nervo periférico (Phan and Schuind 2012).

Alguns **biomateriais** utilizados no desenvolvimento de membranas e tubos-guia utilizados na regeneração de nervo periférico parecem apresentar um efeito promotor na migração e proliferação das células de Schwann, como o **quitosano** que é um polissacarídeo derivado da quitina que apresenta ainda propriedades anti-tumorais e antimicrobianas (Yuan Y 2004, Zheng and Cui 2012).

O uso de **sistemas celulares** capazes de se diferenciarem em células do tipo neurogliais pode melhorar a recuperação sensitiva e motora. Células estaminais mesenquimatosas da medula óssea, tecido adiposo ou do tecido do cordão umbilical, as células estaminais embrionárias, ou mesmo as células estaminais hematopoiéticas da medula óssea são os sistemas celulares mais frequentemente testados e utilizados para promoverem a regeneração do tecido nervoso periférico. Os sistemas celulares implantados nas zonas de lesão nervosa podem produzir fatores de crescimento ou moléculas da matriz extracelular, ou podem modular o processo inflamatório, de forma a melhorar a regeneração nervosa (Luis, Rodrigues et al. 2007, Luís, Rodrigues et al. 2008, Amado, Rodrigues et al. 2010, Simoes, Amado et al. 2010, Gu, Ding et al. 2011, Gartner, Pereira et al. 2012, Gärtner, Pereira et al. 2013, Gartner, Pereira et al. 2014). Alguns autores também aplicaram células neurogliais diferenciadas *in vitro* a partir de células de neuroblastoma, no entanto, com evidentes desvantagens associadas à sua natureza e origem neoplásica (Luis, Rodrigues et al. 2008). Os sistemas celulares podem ser aplicados localmente utilizando um veículo apropriado, como por exemplo um hidrogel, dentro dos tubos-guia que são interpostos entre os topos proximal e distal do nervo ou podem ser infiltrados na área da lesão de esmagamento, nos casos de neurotmese e axonotmese respetivamente. Alternativamente podem ser previamente adicionados ao tubos-guia fazendo o sistema celular crescer *in vitro* em

monocamada sobre o biomaterial a aplicar posteriormente na lesão (Maurício, Luís et al. 2011).

## 3. Cirurgia do Sistema Nervoso Periférico

Quando um nervo é seccionado pode ser reparado de várias formas (Seddon H 1972, Matsuyama, Mackay et al. 2000), sendo os melhores resultados obtidos quando não há perda de substância, pois permite a anastomose primária ou sutura topo-a-topo dos dois topos nervosos. No entanto esta abordagem nem sempre é possível, e quando há perda de substância entre os dois topos nervosos são necessárias diferentes estratégias cirúrgicas a fim de evitar suturas sob tensão (Ijkema-Paassen J 2004). Desde a década de 70 que se iniciou a aplicação de enxertos autólogos de nervo e mais recentemente de tubos condutores que podem ser biológicos (por exemplo, veias) ou sintéticos (tubos guia, de diversos

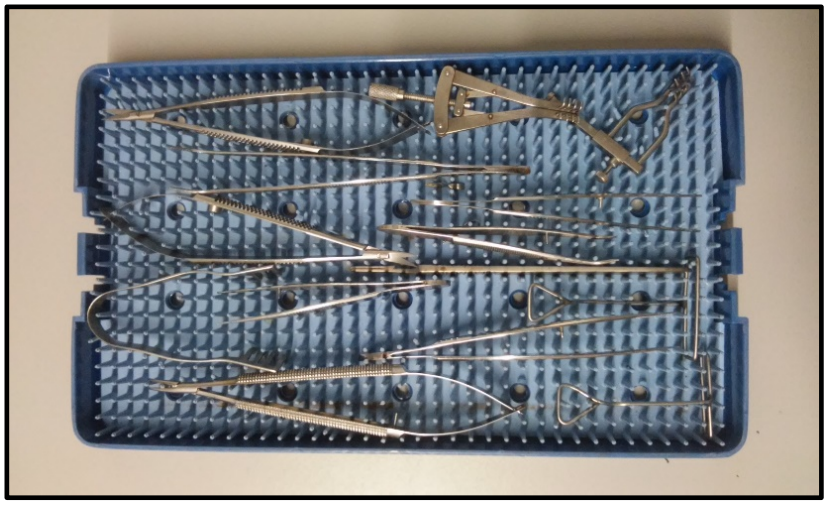

Figura 5 - Caixa de material de microcirurgia

biomateriais e origens) (Pfister, Papaloïzos et al. 2007, Luis, Rodrigues et al. 2008, Pfister, Gordon et al. 2011, Gartner, Pereira et al. 2012, Gärtner, Pereira et al. 2012).

As anastomoses nervosas com ou sem interposição de enxertos autólogos, heterólogos ou tubos-guia condutores da regeneração, são soluções cirúrgicas atualmente utilizadas na cirurgia de nervo periférico que quase sempre implicam a utilização de técnicas e material de microcirurgia e de microscópio cirúrgico ou lupas de aumento (Birch 2011) (Figura 5). O sucesso da reparação nervosa não pode ser apenas avaliado pela recuperação anatómica da estrutura nervosa, mas deve ser acompanhada pela recuperação funcional do nervo, e para tal é fundamental que

sejam estabelecidas as corretas conexões entre axónios motores e sensoriais do topo proximal com os seus equivalentes do topo distal (Brushart T M 1983). A obtenção de melhores resultados está igualmente associada à escolha da técnica cirúrgica mais apropriada para cada lesão, pelo que o cirurgião tem de conhecer e estar familiarizado com os diversos métodos de reparação nervosa à sua disposição (Naff and Ecklund 2001, Ijkema-Paassen J 2004). A tensão no local da sutura diminui o aporte sanguíneo, causando danos nervosos irreparáveis e conduzindo a uma má regeneração, comprometendo deste modo a recuperação funcional (Lundborg and Rydevik 1973, Miyamoto 1979, Clark, Trumble et al. 1992) e aumenta a probabilidade de formação de **neuromas** e aparecimento de **dor neuropática** (Dellon and Mackinnon 1988), principais complicações da anastomose de nervo periférico. O tratamento dos neuromas passa pela excisão cirúrgica do neuroma, com elevado risco de recidiva, apresentando uma eficácia de cerca de 80%. O tratamento médico, através do uso de anti-inflamatórios é geralmente pouco eficaz (Tyner, Parks et al. 2007).

A gravidade da lesão do nervo altera de forma drástica o prognóstico quanto à recuperação morfológica e funcional, sendo influenciado pela distância de afastamentos dos topos nervosos e da presença de algum tipo de material que estabeleça a ponte entre os dois topos (Madison RD 1999). Este facto é de extrema

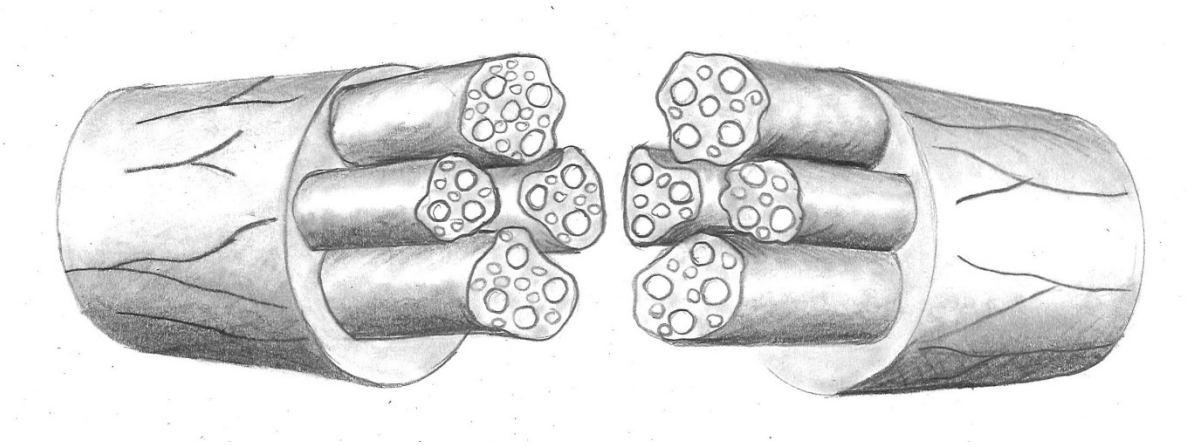

Figura 6 - Lesão de neurotmese com preservação estrutural do nervo periférico (Ilustração de Duarte Monteiro)

importância na escolha da correção clínica, assim como das expectativas de recuperação de cada tipo de lesão, pois em lesões de neurotmese (Figura 6) o uso de suturas topo-a-topo diretas ou uso de enxertos de nervo permitem aos axónios em regeneração encontrarem-se imediatamente em contato com o microambiente da linha de sutura, enquanto na técnica de tubulação os axónios inicialmente

regeneram para o espaço vazio, o que resulta num menor número de axónios motores a entrar em contacto com o órgão efetor (Madison RD 1999). Já em lesões de axonotmese ou de neuropraxia, a manutenção da lâmina basal das células de Schwann servem de guia aos axónios em regeneração, o que permite uma maior precisão da regeneração, permitindo que maior número de axónios atinjam o órgão alvo (Witzel C 2005). No caso das lesões de neurotmese, seria lógico pensar que a especificidade da reinervação sensitiva/motora fosse aumentada com um maior alinhamento dos topos nervosos, por forma a evitar a dispersão das fibras sensoriais e motoras no local da lesão. Para tal, existem diversas técnicas de neurorrafia possíveis:

- **Sutura topo-a-topo clássica ou epineural (epineuro-epineuro)**
- **Sutura inter-fascicular**
- **Sutura oblíqua**
- **Sutura terminolateral**
- **Utilização de cola de fibrina**

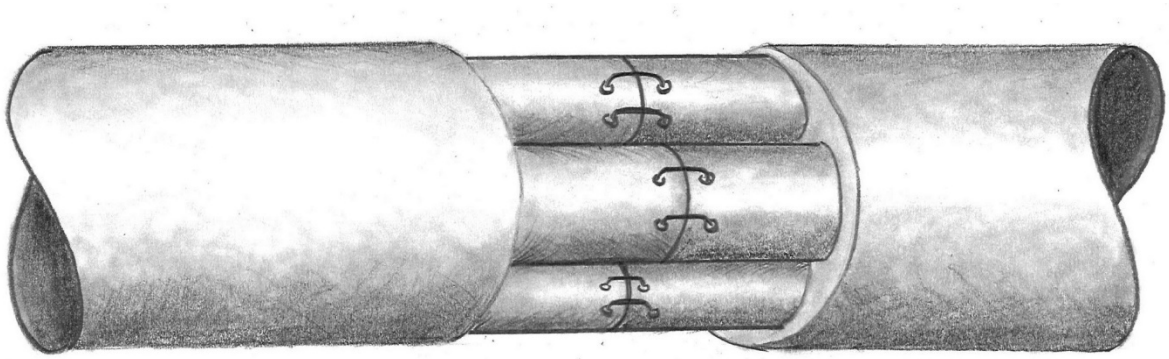

Figura 7 - Sutura interfascicular (Ilustração de Duarte Monteiro)

A evolução de técnicas de microcirurgia, assim como da bioengenharia, tem disponibilizado aos cirurgiões diferentes técnicas de resolução das lesões de nervo periférico que apresentam afastamento dos topos nervosos:

- **Interposição de autoenxertos**
- **Aplicação de *flaps* epineurais**
- **Técnica de tubulação**
- **Utilização de fatores de crescimento**
- **Utilização de sistemas celulares capazes de promover a regeneração nervosa**

- **Combinação entre as técnicas anteriores**

O cirurgião, conhecendo as diferentes opções pode optar pela que considere a melhor alternativa para cada caso, podendo ainda utilizar combinações de diferentes técnicas, otimizando assim os resultados.

As técnicas de neurorrafia mais básicas são a **técnica epineural** e a **técnica interfascicular.** É ainda controversa a vantagem de uma das técnicas relativamente à outra, pois ambas apresentam vantagens e desvantagens. A **técnica epineural** é tecnicamente mais simples e rápida de realizar, implica uma menor manipulação tecidual (permitindo melhor preservação das estruturas internas do nervo assim como do seu suprimento sanguíneo) (Matsuyama, Mackay et al. 2000). A **técnica interfascicular** permite um melhor alinhamento entre os respetivos tubos endoneurais, mas, para que tal seja possível, é necessário a distinção dos feixes motores e sensitivos, sob pena de se obter um alinhamento impróprio que é ainda mais prejudicial (Millesi 2006). No entanto, esta técnica tem como principais desvantagens a formação de uma cicatriz no local da sutura, potencial dano das fibras interfasciculares (pela manipulação cirúrgica) e interrupção do suprimento sanguíneo, é mais morosa e tecnicamente mais complicada de executar (Matsuyama, Mackay et al. 2000).

Em ambas as técnicas se podem obter bons resultados, no entanto o cirurgião deve ter em conta que a presença de suturas demasiado fechadas pode ser responsável por alinhamentos impróprios (Millesi 2006), e com isso, maus resultados funcionais.

Uma outra opção é o uso de **cola de fibrina**, que apresenta como principais vantagens o facto de ser uma técnica mais simples e rápida de executar, capaz de promover a regeneração nervosa, uma menor resposta inflamatória local e tem uma menor interferência na microcirculação local (Martins, Siqueira et al. 2005). A principal desvantagem prende-se com o facto de a sua aplicação estar limitada a nervos de pequeno calibre (Martins, Siqueira et al. 2005).

A anastomose topo-a-topo com seccionamento do nervo a 90º é a técnica clássica, mas alguns autores (Kotulska, Marcol et al. 2006) concluíram que quer a sutura obliqua direta quer a sutura oblíqua com interposição de um enxerto, quando realizada com um ângulo de 30º produz uma melhor recuperação quer funcional quer morfológica. Neste estudo, o número de axónios mielinizados e o seu diâmetro

no topo distal assim como o número de células de Schwann eram maiores nos grupos com anastomoses realizadas com o ângulo de 30º, quando comparados com a técnica clássica. A fibrose epineural e intraneural era significativamente menor nas anastomoses realizadas a 30º, o que é importante, pois o excesso de tecido conectivo pode dificultar mecanicamente a progressão da regeneração. Neste estudo, a avaliação funcional é consistente com os resultados morfológicos obtidos (Kotulska, Marcol et al. 2006).

Existem no entanto outras técnicas que tal como a anterior visam o objetivo de aumentar a superfície de contacto entre os dois topos, como é o caso da interposição de um anel metálico externo ao epineuro (Kayıkçıoğlu, Karamürsel et al. 2004).

Os casos de lesão nervosa mais complicados são, como já referido, as situações em que a neurotmese é acompanhada com perda de tecido nervoso, que impede a neurorrafia direta ou que esta seja efetuada com excesso de tensão. Alguns autores comprovaram que a sutura topo-a-topo é a técnica que apresenta melhores resultados, mas unicamente quando é passível de ser realizada sem tensão na linha de sutura (Smith and Robinson 1995, Maeda T 1999).

O recurso a enxertos, nomeadamente autólogos, tem sido amplamente utilizado como um dos métodos de eleição para reparação cirúrgica, em caso de perda de tecido nervoso, sendo ainda considerado o *Gold Standard*. No entanto, este método apresenta também várias desvantagens (Millesi H 1990, Madison RD 1992, Dahlin and Lundborg 1998, Flores AJ 2000, Lee AC 2003, Varejão. 2003, Udina E 2004, Bellamkonda 2006, Tyner, Parks et al. 2007):

- Necessidade de uma segunda intervenção cirúrgica e a morbilidade causada no local dador
- O diâmetro do enxerto de nervo autólogo é frequentemente mais pequeno;
- Reinervação frequentemente insatisfatória
- Risco de necrose do enxerto autólogo ao longo da sua extensão, devido a má vascularização
- Surgem alterações de natureza sensorial
- Formação de neuromas e de tecido fibroso junto à segunda linha de sutura

Em casos de enxertos autólogos e em alotransplantes, de forma a evitar o máximo de morbilidade no local da colheita, usam-se normalmente nervos sensitivos como: o nervo sural, o nervo grande auricular, o ramo anterior do nervo antebraquial medial, o ramo cutâneo dorsal do nervo ulnar, o ramo sensorial superficial do nervo radial (Matsuyama, Mackay et al. 2000, Pogrel 2002). É sabido que a utilização de nervos sensitivos não promove uma ótima regeneração de axónios motores, quando comparada com a regeneração induzida por nervos mistos (Nichols CM 2004). O que poderá ser uma razão para maus resultados. Apesar das técnicas de microcirurgia utilizadas após reparação de nervos periféricos destruídos, lesados ou em reconstruções dos plexos braquiais, os resultados não são dececionantes mas são piores do que se gostaria, devido a atrofias musculares que surgem durante o período de desinervação. Desde o início do século XX que existem ensaios e experiências tentando ultrapassar estas limitações. O conceito de **tubulação** envolve o uso de um canal oco, construído em material natural ou sintético, designado por tubo-guia, que é interposto nos segmentos lesionados. Durante as

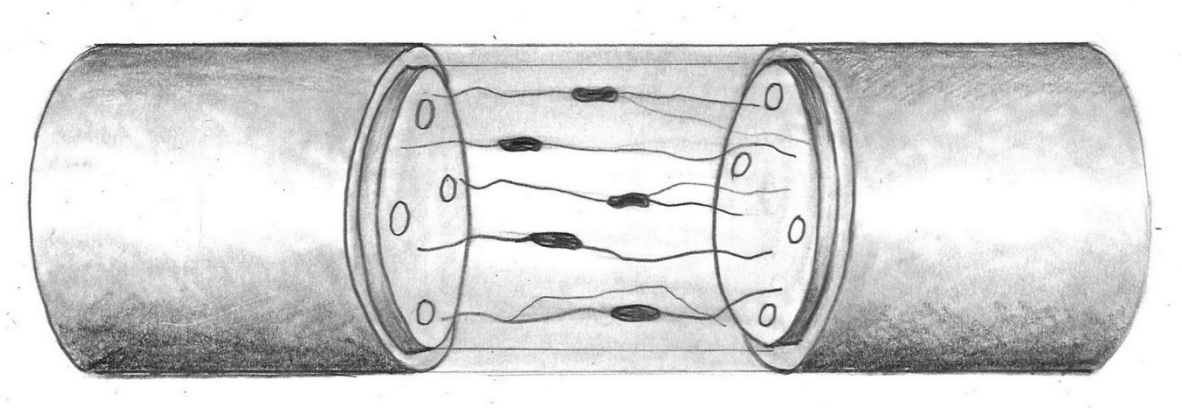

Figura 8 - Técnica de tubulação (Ilustração de Duarte Monteiro)

últimas décadas, e no sentido de se ultrapassarem as desvantagens encontradas, especialmente com a utilização dos enxertos autólogos, diversos laboratórios têm investigado a aplicação de tubos de diferentes biomateriais em lesões de nervo periférico, que se baseia na orientação das fibras nervosas (denominado fenómeno de quimiotropismo) permitindo que as fibras do topo proximal se encontrem com as do topo distal, através de sinais químicos originados no topo distal (Hundson TW 2000). A utilização de biomateriais para fabrico de tubos-guia tem sido alvo de grande interesse e muitos autores têm realizado investigação neste sentido, incluindo o grupo de investigação em que se insere o trabalho desta dissertação (Lundborg G 1982, Gibson KL 1991, Madison RD 1992, Meyer RS 1997, Steuer H

1999, Hundson TW 2000, Lee WP 2000, Heijke GCM 2001, Meek M F 2002, Lee AC 2003, Varejão. 2003).

Na técnica de tubulação, ambos os topos nervosos são introduzidos dentro do tubo-guia, fazendo este a ponte entre os dois topos. Ambos os topos nervosos são fixados ao tubo-guia através de sutura com fio monofilamentar não absorvível de calibre 7-0 a 9-0, com 2 a 3 pontos simples entre o tubo-guia e o epineuro, permitindo a regeneração, por crescimento do topo proximal em direção ao topo distal (Stoll G 2002).

Baseadas na técnica de tubulação, alguns autores têm desenvolvido outras abordagens, como a **técnica do *flap* epineural** (Karacaoğlu, Yüksel et al. 2001, Siemionow, Tetik et al. 2002, Tetik, Ozer et al. 2002, Ignatiadis, Yiannakopoulos et al. 2007, Siemionow and Brzezicki 2009), que consiste em efetuar o desbridamento cirúrgico do epineuro sem lesar os fascículos nervosos subjacentes, removendo-o completamente ou não e fazendo deslizar a manga criada suturando-a ao outro topo nervoso. Regra geral, os resultados são melhores quando é utilizado o epineuro do topo proximal. Esta técnica necessita da interposição, ainda que possa ser temporária (durante a cirurgia), de um biomaterial que forneça sustentação ao tubo de epineuro, para que este não colapse e seja possível a realização da técnica. O colapso do lúmen após a remoção do tubo de biomaterial usado para sustentação, parece estar associado aos maus resultados obtidos quando esta técnica é aplicada em grandes defeitos nervosos (Ignatiadis, Yiannakopoulos et al. 2007). Por esta razão e por se tratar de uma técnica de difícil execução e que aumenta muito o tempo de cirurgia, não tem sido muito explorada.

## 4. Métodos de Avaliação de Regeneração e Recuperação funcional

A experimentação animal em regeneração de nervo periférico é geralmente realizada em nervo ciático de rato (Dellon and Mackinnon 1988). A pesquisa nesta área necessita de combinar a avaliação da recuperação quer a nível funcional quer a nível morfológico. A avaliação funcional é mais difícil de obter de forma objetiva, não existindo um único método consensual de avaliação, sendo por isso recomendados diversos métodos de avaliação funcional, utilizados por diversos investigadores nesta área (Morris 1972, Varejão ASP 2001, Varejão ASP 2001, Varejão ASP 2004, Luís 2008, Luis, Rodrigues et al. 2008) (Figura 9):

- Reação postural de extensão (RPE)
- Avaliação sensorial (WRL)
- Avaliação da pegada
    - Índice de funcionalidade do ciático (SFI)
    - Funcionalidade do ciático em condições estáticas (SSI)

Entre os diversos testes de avaliação motora e nociceptiva, os testes **de reação postural de extensão (RPE)** (Thalhammer, Vladimirova et al. 1995) e de avaliação sensorial (WRL) respetivamente, provaram ser métodos fiáveis, válidos e altamente eficientes na determinação da recuperação funcional após lesão nervosa do nervo ciático (Gärtner, Pereira et al. 2013).

O RPE é um teste funcional de fácil execução e permite uma avaliação motora mesmo quando não é possível a avaliação da pegada (regra geral, por autotomia),com resultados comparáveis a esta última.

A **avaliação sensorial (WRL)** é um teste de avaliação funcional sensitiva que se baseia na quantificação do reflexo flexor quando sujeito a uma estimulação térmica (Hu, Hu et al. 1997, Varejão ASP 2003).

Ambos os testes dependem da sensibilidade do operador, devendo ser sempre realizado pelo mesmo operador, na mesma sala, na mesma sequência, num

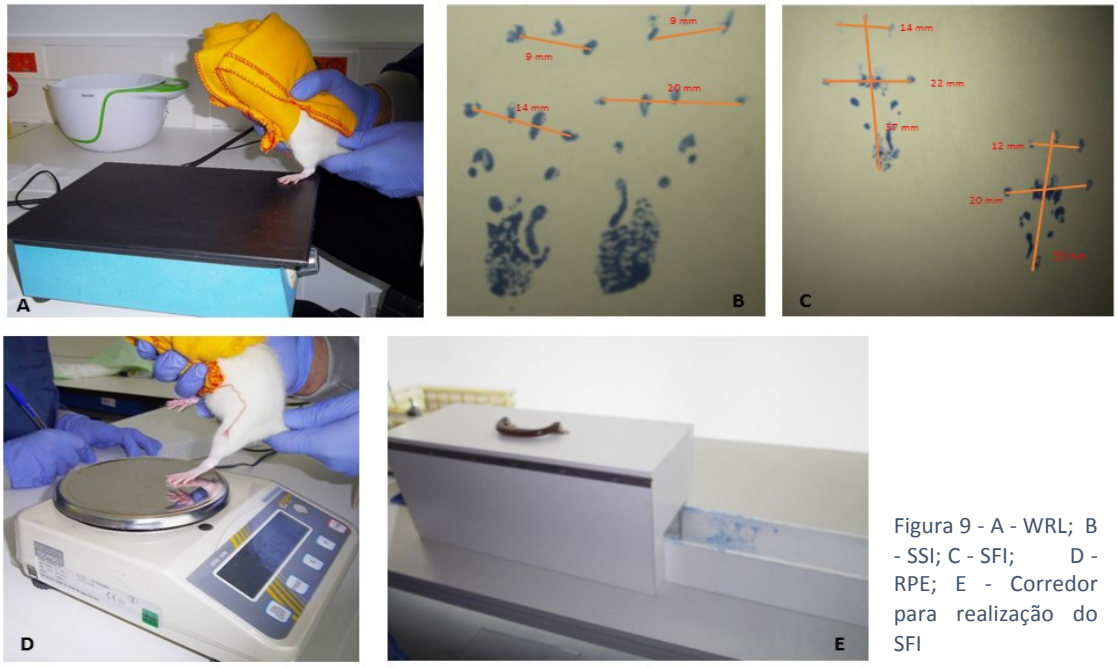


Figura 9 - A - WRL; B - SSI; C - SFI; D - RPE; E - Corredor para realização do SFI

ambiente calmo e sempre com o mesmo material, para se evitar variações entre leituras (Hadlock, Koka et al. 1998, Koka R 2001).

O **estudo da pegada (*Walking Track analysis*)** é um método de quantificação da recuperação funcional do nervo ciático desenvolvido por De Medinaceli e seus colaboradores (De Medinaceli L 1982), cujo modelo é o rato. A partir deste estudo o **Índice de Funcionalidade do Ciático (SFI)** passou a ser utilizado como ferramenta na avaliação funcional das lesões do ciático em ratos, com resultados precisos e fiáveis (Bain, Mackinnon et al. 1989, Varejão ASP 2003, Varejão ASP 2004, Nichols, Myckatyn et al. 2005, Luís, Amado et al. 2007, Luis, Rodrigues et al. 2007, Luis, Rodrigues et al. 2007, Luis, Rodrigues et al. 2008). No entanto, em algumas circunstâncias a medição do comprimento da pegada não era possível medir, pelo que um outro método de avaliação da pegada foi proposto por Bervar (Bervar M 2000) denominado **Índice de Funcionalidade do Ciático em Condições Estáticas (SSI)** (Bervar M 2000), passando ambos a ser utilizados de forma corrente nos estudos de lesão de nervo periférico (Varejão ASP 2003, Varejão ASP 2004, Nichols, Myckatyn et al. 2005, Luís, Amado et al. 2007, Luis, Rodrigues et al. 2007, Luis, Rodrigues et al. 2007, Luis, Rodrigues et al. 2008, Gartner, Pereira et al. 2012, Gärtner, Pereira et al. 2012, Gartner, Pereira et al. 2014). Os estudos de regeneração de nervo periférico são, regra geral, e desde 1982, realizados em diversas estirpes de *Rattus norvegius,* pelo que desde então tem sido estudada a impressão da sua pegada para cálculo de dois índices que caracterizam a funcionalidade do nervo ciático – o SFI e o SSI. Os valores do SFI e do SSI variam entre zero, que corresponde a uma função plenamente normal, e -100 no caso de uma disfunção completa do membro experimental (Kanaya, Firrell et al. 1996, Terris, Cheng et al. 1999, Bervar M 2000). O SSI foi proposto por Bervar (Bervar M 2000), como resposta às limitações do SFI tais como a dificuldade de obtenção de pegadas de qualidade (Bain, Mackinnon et al. 1989, Hare GMT 1992, Varejão ASP 2001), a subjetividade da avaliação (Grasso G 2004), a marcha do animal a velocidade não constante ou interrompida (Dellon and Dellon 1991, Dijkstra, Meek et al. 2000), o aumento de peso dos animais ao logo do período experimental (Dellon and Dellon 1991), a compensação por parte do membro normal (Chamberlain, Yannas et al. 2000, Dijkstra, Meek et al. 2000, Meek MF 2001), o aparecimento de contraturas musculares (Kobayashi, Mackinnon et al. 1997, Chamberlain, Yannas et al. 2000,

Watson, Jejurikar et al. 2001, Sagiv, Shabat et al. 2002) ou a autotomia (Zellem, Miller et al. 1989, Navarro, Verdú et al. 1994, Hadlock, Koka et al. 1998, Kauppila 1998, Rodriguez JF 1999, Luis, Rodrigues et al. 2007). A explicação dos testes anteriormente referidos encontra-se descrita no trabalho experimental desta dissertação, pelo que optamos por omiti-la nesta secção.

A avaliação funcional através dos testes referidos previamente apesar de extremamente úteis, apresentam como vimos algumas limitações e fragilidades, pelo que a **análise cinemática da marcha** é uma ferramenta de grande utilidade, sendo cada vez mais o método de avaliação do movimento mais utilizado (Knudson and Morrison 1997) para avaliação, monitorização e diagnóstico (Winter 2009). A sua utilização foi sendo aprimorada ao longo do tempo, através do desenvolvimento tecnológico, o que permite a obtenção de mais informação cinemática. Para que tal acontecesse, foi fundamental o desenvolvimento de câmaras de vídeo de alta velocidade e computadores e programas informáticos sensíveis capazes de detetar pequenas alterações, permitindo uma melhor compreensão de patologias quer ortopédicas quer neurológicas (Rowe, Durward et al. 1999) em diversas espécies (McLaughlin 2001, Huitema, Hof et al. 2002). Por esta razão iremos aprofundar um pouco mais a descrição deste método ao longo deste texto.

A compreensão da análise da locomoção do rato permite uma análise biomecânica, que implica coordenação entre receção sensorial, resposta motora e integração cortical, sendo cada vez mais utilizada em estudos de lesões de nervo periférico e musculares (Varejão. 2003). A **cinemática** é uma técnica de avaliação do movimento, que descreve os detalhes do movimento em si mesmo, independentemente das forças, internas e externas, que o originam (Winter 2009), e envolve variáveis como o deslocamento linear e angular, velocidade e aceleração (Nigg and Herzog 2007, Winter 2009). A recuperação da atividade locomotora após lesão do sistema nervoso, surgiu como um dos maiores desafios da neurociência clínica. Muitos pacientes com lesões neurológicas, quer de nervo periférico quer da medula espinal, sofrem de fraqueza muscular e perda de controlo independente das articulações, resultando em alterações da marcha. Nos últimos anos, muito se desenvolveu em relação à análise da marcha do rato o que alterou significativamente a investigação em lesões e regeneração de nervo periférico (Costa, Simoes et al. 2009). De facto, o uso de parâmetros biomecânicos permitiu

uma valiosa compreensão dos efeitos da desenervação/reinervação do nervo ciático e representou uma integração do controlo neural que atua no tornozelo e musculatura do pé (Varejão, Cabrita et al. 2003, Varejão ASP 2003, Varejão ASP 2004).

O estudo cinemático da marcha do rato para avaliar a recuperação / regeneração do nervo ciático foca-se essencialmente na articulação tibio-társica, pois o movimento angular desta articulação sofre alterações significativas nas lesões de axonotmese e de neurotmese deste nervo misto, assim como durante a sua recuperação funcional (Varejão ASP 2003).

A análise cinemática necessita que os animais se movimentem em linha reta, pelo que eles percorrem um corredor com paredes de acrílico e o movimento é captado por uma câmara de alta resolução, sendo o movimento analisado posteriormente usando um programa informático apropriado como está detalhadamente descrito no trabalho experimental desta dissertação (Varejão ASP 2001, Varejão, Cabrita et al. 2003, Amado, Simoes et al. 2008) (Figura 10).

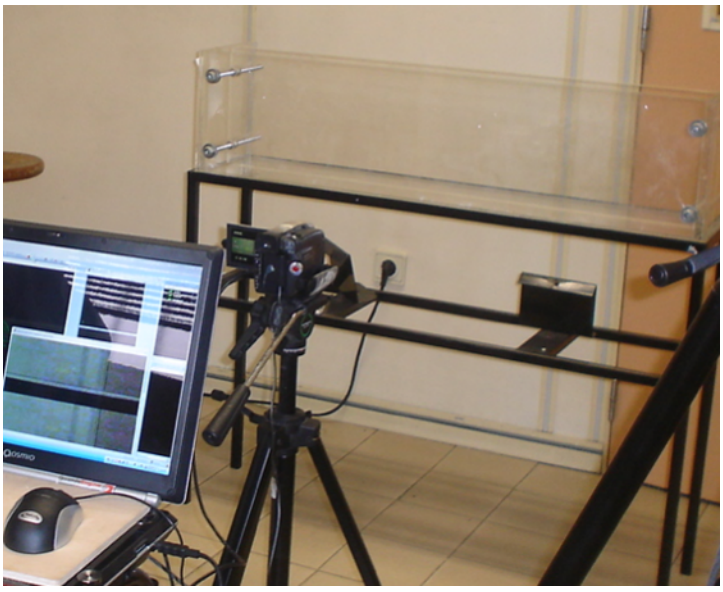

Figura 10 - Mecanismo para obtenção de imagens para avaliação cinemática (Fotografia gentilmente cedida pela Professora Doutora Sandra Amado)

A análise biomecânica 2D (plano sagital ou bidimensional) é realizada aplicando um modelo de dois segmentos à articulação do tornozelo, adotado do modelo desenvolvido por Varejão e colaboradores (Varejão, Cabrita et al. 2003). Neste modelo, são tatuados três pontos com tinta preta, na extremidade proximal da tíbia, no maléolo lateral e na cabeça do 5º metatarso. Estes pontos permitem definir dois

segmentos rígidos: um que une o joelho ao maléolo (segmento da perna) e outro

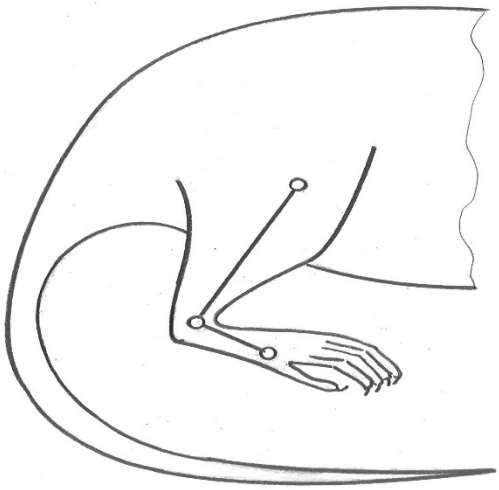

Figura 11 - Análise cinemática através da avaliação do movimento angular (Ilustração de Duarte Monteiro)

que une o maléolo à cabeça do 5º metatarso (Figura 11). Neste modelo, valores positivos e negativos da posição da articulação do calcanhar (Θº) indicam flexão dorsal e flexão plantar do pé, respetivamente. No rato o pé e a perna encontram-se em posição neutra, praticamente em ângulo reto, sendo que nesta posição o valor do ângulo da articulação tibiotársica é de 90º (Varejão, Cabrita et al. 2002, Amado 2007).

A análise cinemática, apesar de ser uma avaliação objetiva da marcha, pode no entanto estar sujeita a alguns erros. Um dos problemas deste tipo de avaliação prende-se com o facto de existir mais de um modelo biomecânico bidimensional descrito na literatura, especialmente relacionado com a definição dos segmentos rígidos (Metz, Merkler et al. 2000, Yu, Matloub et al. 2001, Varejão, Cabrita et al. 2002). Um outro problema frequentemente referido na bibliografia prende-se com o facto de o movimento da pele na região da articulação do joelho influenciar a localização da zona articular (Metz, Merkler et al. 2000, Basso 2004, Filipe, Pereira et al. 2006, Nigg and Herzog 2007). Outros problemas podem resultar em erros na determinação da distância entre marcas de coordenadas, como o sistema da câmara (erro das lentes), distância entre o corredor e a câmara (com um plano de movimento diferente do plano calibrado), erros de projeção (no caso de posição obliqua da câmara relativamente ao plano do movimento). Todos estes problemas podem resultar em erros de dois tipos (Nigg and Herzog 2007):

- Erro na determinação das coordenadas das marcas de calibração;
- Erro na representação dos segmentos cinemáticos usando informação das marcas;

No entanto têm sido sugeridas novas abordagens para corrigir o erro relacionado com o movimento da pele, como o método invasivo das marcas (Nigg and Herzog 2007).

O **deslocamento angular** da articulação tibiotársica durante a fase de suporte é definido como a mudança na posição angular (Robertson, Caldwell et al. 2013), medida em graus, e a sua análise durante a fase de suporte é de extrema importância dado ser nesta fase que se dá a atividade contrátil dos músculos posteriores da perna, oferecendo resistência à flexão dorsal e originando uma flexão plantar ativa (Varejão, Cabrita et al. 2003).

O **fator de suporte** define o rácio entre a duração de contacto com o chão do membro experimental e do membro normal, durante a marcha (Walker, Resig et al. 1994), que deve ser próximo dos 100% em membros sem lesão e apresenta valores inferiores para membros com lesão do nervo ciático.

A **velocidade da marcha** é um parâmetro que influencia a duração das fases da locomoção (apoio e suspensão) (Gillis GB 2002), sendo expressa em centímetros por segundo (cm/s).

A **duração da fase de suporte** consiste no intervalo de tempo (expressa em milissegundos (ms)) em que o membro está em contacto com o solo (Craik and Oatis 1995). Em membros com lesão do nervo ciático há um encurtamento da fase de suporte, atribuído ao decréscimo da força muscular apresentada nesse membro (Yu, Matloub et al. 2001).

A duração de cada fase da marcha é um parâmetro que compara o tempo de suporte com o tempo de suspensão do membro experimental e posteriormente compara com o mesmo rácio do membro normal (Yu, Matloub et al. 2001).

O **comprimento da passada** é definida como a distância entre a cabeça do metatarso médio de um pé do membro pélvico e o outro (Yu, Matloub et al. 2001), medida em milímetros (mm) e parece ser influenciada pela velocidade (Hoyt, Wickler et al. 2000).

O **ângulo de rotação externa do dedo (*Toe out angle*)** é definido como o ângulo entre o eixo longitudinal do pé (desde o calcâneo até à extremidade do terceiro dedo) e a linha de progressão do animal (Varejão ASP 2003).

A cinemática individual da articulação tibiotársica quer em grupos controlo quer em animais com lesão nervosa é caracterizada por alta variabilidade quer entre animais quer entre diferentes passos do mesmo animal, característica intrínseca do movimento de animais quadrúpedes (Jacobson and Guth 1965) pelo que foi necessário parametrizar o movimento, tendo Varejão e colaboradores (Varejão, Cabrita et al. 2002) definido para cada ciclo de apoio do membro, quatro fases:

1. **Contacto inicial (IC**) – a articulação tibiotársica (TT) encontra-se em flexão plantar e o apoio é feito no solo através dos dedos. Sendo esta uma fase de posicionamento do membro a posição articular determina o padrão de resposta do membro ao suporte de peso (Perry, Burnfield et al. 1992);
2. **Saída da parte distal do pé contra-lateral (OT)** – o período entre o contacto inicial (IC) e a saída da parte distal (OT) do pé contra-lateral e corresponde aos primeiros 20% da fase de suporte, em que após a flexão plantar da articulação tibiotársica, as almofadas distais e proximais entram em contacto com o solo. Nesta fase, designada por **transferência de suporte,** termina o apoio duplo, as principais funções são a absorção do impacto do solo, a estabilidade no suporte do peso e preservar a progressão (Perry, Burnfield et al. 1992);
3. **Saída do Calcanhar (HR)** – o período entre a saída da parte distal do pé contra-lateral (OT) e a saída do calcanhar do membro de apoio (HR) corresponde aos segundos 20% da fase de suporte, designada **fase intermédia de suporte**, em que se regista um movimento da tíbia no sentido cranial, sobre o pé, que permanece apoiado na sua superfície plantar, enquanto a que a articulação tibiotársica passa de uma flexão plantar para uma flexão dorsal, apresentando um ângulo da articulação tibiotársica de aproximadamente 90º, sendo o principal objetivo desta fase, assegurar a progressão sobre o pé de apoio e a estabilidade do membro (Perry, Burnfield et al. 1992);
4. **Saída da parte distal do pé (TO)** – o período entre HR e TO corresponde aos restantes 60% finais da fase de suporte. Corresponde ao último parâmetro do intervalo de apoio num membro, em que se verifica uma progressão do corpo para a frente do pé, o animal começa a elevar o

calcanhar e o membro posiciona-se para entrar na fase de suspensão (Perry, Burnfield et al. 1992);

A redução da subjetividade e da variabilidade tem sido e será o principal desafio na avaliação funcional em lesões do nervo periférico. Neste sentido, alguns autores têm apresentado algumas soluções quer para melhorar algumas das técnicas já descritas quer apresentado novas.

Relativamente à análise cinemática, por forma a homogeneizar a velocidade da passada alguns autores propuseram a utilização de uma esteira rolante (Gärtner, Maurício et al. 2013) que embora reduza a variabilidade entre passos do mesmo animal apresenta como principais problemas, o custo do equipamento e limita a possibilidade de combinar outros dados cinemáticos como as forças de reação ao solo. Outras possibilidades passam pela análise cinemática de todo o membro pélvico (Jacobson and Guth 1965, Pereira, Maurício et al. 2013), ou a análise e reconstrução tridimensional procurando a morfologia do movimento e do movimento de cada segmento (Amado 2007).

Outros testes utilizados por diversos investigadores no estudo da recuperação funcional de lesões de nervo periférico são as forças de reação ao solo, utilizando uma plataforma de forças (Howard CS 2000), eletromiografia (Meek MF 2001, Maurício, Gärtner et al. 2011), performance da locomoção (Meek MF 2001), estudos de função proprioceptiva (Hadlock, Koka et al. 1998), quantificação da reinervação das glândulas sudoríparas da face plantar do pé (Rodríguez, Gómez et al. 1999, Verdú, Ceballos et al. 2000, Vilches and Navarro 2000), recuperação da dor (Rodríguez, Gómez et al. 1999) e pesagem dos músculos gastrocnémios e solear (Varejão. 2003).

Atualmente, a **análise morfológica** é o método mais comum de estudo de regeneração nervosa (Raimondo, Fornaro et al. 2009) e a investigação da morfologia do nervo pode conferir informação de extrema importância no processo de regeneração e correlação com a recuperação funcional (Geuna, Raimondo et al. 2009). A análise morfológica permite avaliar a presença ou ausência de fibras nervosas no nervo periférico reparado, a distribuição espacial dessas fibras e a sua quantidade, a existência, ausência e qualidade da mielinização, a presença e avaliação do tipo de tecidos vizinhos assim como a verificação da reabsorção total ou parcial dos biomateriais utilizados. A análise quantitativa das fibras nervosas é

fundamental nos estudos de reparação de nervo periférico (Geuna S 2000, Geuna, Tos et al. 2001, Geuna, Raimondo et al. 2009).

A histomorfometria permite-nos obter uma ideia mais clara sobre o que se está a passar em termos citológicos durante o processo de regeneração, pelo que a avaliação quer da quantidade quer da qualidade das fibras nervosas que se formam no local da lesão é de extrema importância. Em 2000, Geuna e seus colaboradores estabeleceram um método com regras, tendo em conta a especificidade da organização do nervo periférico, definindo a **regra de igualdade de oportunidade** que assegura que todas as fibras nervosas têm a mesma oportunidade de serem incluídas na amostra, através de um conjunto de regras de amostragem, o *design-based sampling*, de onde resulta uma **amostragem aleatória sistemática** (Geuna S 2000). Este método consiste na avaliação de pelo menos 15 campos de menores dimensões (em vez de um menor número de campos de maiores dimensões (Schmitz 1998), em que o primeiro campo é escolhido de forma aleatória e a parir dele se selecionam os outros campos, através da sistematização de um processo de salto para o campo seguinte, localizado a uma distância fixa (Geuna S 2000).

Igualmente, uma das especificações do nervo periférico consiste na possibilidade de a mesma fibra nervosa de maiores dimensões seja lida em mais de um campo e lida mais de uma vez (Geuna, Tos et al. 2001) – **efeito de margem**, pelo que mais uma vez, Geuna e os seus colaboradores (Geuna S 2000) desenvolveram o método de **disseção bi-dimensional** – ***two-dimensional dissector*****,** em que é assinalado o topo de cada fibra e somente estas são lidos e no sentido norte/sul, permitindo que cada fibra apenas seja lida uma vez. Este ***two-dimensional dissector*** é usado para estimar o número total de fibras mielinizadas (N), densidade, o diâmetro médio das fibras (D), do axónio (d), espessura de mielina (M), rácio espessura de mielina/diâmetro do axónio (M/d), rácio diâmetro do axónio/diâmetro da fibra (d/D – g-ratio).

Em **microscopia ótica** a coloração mais frequentemente utilizada é a hematoxilina-eosina e é utilizada normalmente para uma análise unicamente qualitativa, enquanto a histomorfometria permite também uma avaliação quantitativa e portanto mais objetiva e passível de comparações mais precisas.

A **técnica de imunohistoquímica** baseia-se na utilização de anticorpos que se ligam especificamente a um determinado antigénio celular e que se tornam visíveis ao microscópio por fluorescência ou de laser confocal, permitindo detetar axónios

em regeneração e a possível migração das células de Schwann dentro dos tubos-guia ou nos biomateriais durante o período de regeneração do nervo periférico através do uso dos anticorpos anti-NF-200kd e anti-GFAP respetivamente (Geuna, Tos et al. 2001, Varejão. 2003). Existem atualmente diversas técnicas e colorações, antigénios e anticorpos disponíveis, no entanto a sua utilização tem de ser aferida para cada situação em particular (Luís 2008).

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## Capítulo II – Engenharia de Tecidos

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### 1. Estado da arte

As lesões de nervo periférico são normalmente provocadas por eventos traumáticos acidentais ou iatrogénicos, sendo que as lesões traumáticas de nervo periférico uma causa de significativa morbilidade e incapacidade. As lesões de extremidades podem ser óbvias na sua localização, mas a sua severidade e estruturas subjacentes envolvidas nem sempre são evidentes. (Taylor, Braza et al. 2008).

Estudos anteriores realizados no Canadá e Austrália, em medicina humana, estimaram que 2-3% de todos os pacientes em unidades de traumatologia apresentavam lesão de nervo periférico (Selecki, Ring et al. 1982, Noble, Munro et al. 1998), no entanto, se considerarmos as lesões de plexos e raízes nervosas a incidência pode atingir os 5% (Noble, Munro et al. 1998, Huang and Huang 2006). Estando em medicina humana estimados mais de 500.000 novos pacientes por ano (Rodriguez JF 2004), número este ainda por estabelecer em medicina veterinária. Em medicina humana as lesões são mais frequentes em homens com idades compreendidas entre os 18 e 25 anos, e afetam mais o membro superior (Nichols CM 2004). Está relatado que os nervos mais frequentemente lesados em traumatismos do membro superior são (por ordem de frequência) o radial, ulnar e mediano (Selecki, Ring et al. 1982, Noble, Munro et al. 1998), enquanto no membro inferior são por ordem decrescente de frequência o ciático, peroneal e tibial/femoral (Robinson LR 2005). No entanto, a prevalência de lesão do nervo ciático após fratura acetabular é de 10-13%, especialmente quando há lesão das colunas posteriores do acetábulo. Em medicina veterinária, apesar de estas prevalências não estarem estabelecidas, a experiência clínica em animais de companhia permitem-nos afirmar que as lesões de nervo periférico mais frequentes são as lesões do plexo braquial, do nervo ciático aquando de fratura acetabulares, enquanto lesões de outros nervos são dependentes do tipo e severidade de fraturas de úmero, rádio, cúbito, fémur, tíbia e perónio.

A medicina regenerativa aplicada à reparação e regeneração do nervo periférico é neste momento uma combinação de várias áreas de conhecimento que conjugadas aumentam a possibilidade de êxito na regeneração de nervo periférico

cuja recuperação completa apenas é atingida ocasionalmente e é regra geral insatisfatória (Battiston, Papalia et al. 2009, Siemionow and Brzezicki 2009). A sua recuperação é melhor quando se mantem a continuidade do nervo ou quanto melhor for a sua reconstrução cirúrgica (Geuna, Raimondo et al. 2009, Geuna, Fornaro et al. 2010, Raimondo, Fornaro et al. 2011). Para tal, é hoje consensual que o melhoramento dos dispositivos médicos e das técnicas de microcirurgia não são por si só suficientes para se obter uma total recuperação funcional dos nervos lesados, mas a medicina regenerativa pensada de um ponto de vista multidisciplinar permite atingir um maior nível de inovação, passando por áreas como a microcirurgia reconstrutiva, transplante (órgãos, tecido, células ou genes), bioengenharia (desenvolvimento de novos biomateriais), fisioterapia, bioquímica e farmacologia (Figura 12) (Geuna, Gnavi et al. 2013).

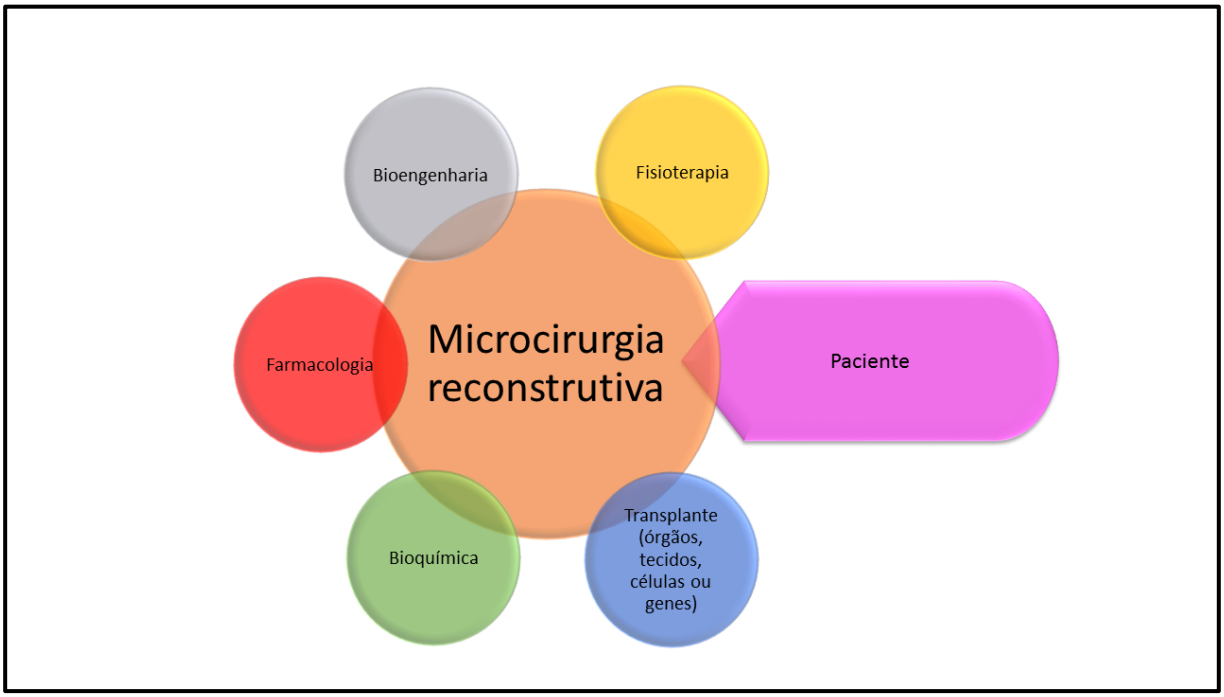



**Figura 12 - Fluxograma de Medicina Regenerativa**

A microcirurgia é a disciplina chave da interação acima descrita, pois regra geral, é o cirurgião que faz a implantação no paciente das diferentes dispositivos pelo que este tem de participar ativamente nos diferentes passos do desenvolvimento tecnológico experimental por forma a se otimizarem todas as etapas e todos os processos (Battiston, Papalia et al. 2009).

A **reconstrução cirúrgica** é já uma abordagem utilizada há alguns anos (Battiston, Papalia et al. 2009), no entanto nas últimas décadas surgiram alguns avanços (Siemionow and Brzezicki 2009). Como foi descrito no capítulo anterior, a

reconstrução microcirúrgica nervosa inclui técnicas mais correntes como a neurorrafia topo-a-topo, e outras técnicas menos correntes como a utilização de autoenxertos, aloenxertos (Teboul, Kakkar et al. 2004, Tung and Mackinnon 2010, Zhang and Gu 2011) ou tubos-guia quando há perda de substância nervosa (Siemionow and Brzezicki 2009), sendo esta última a área onde maiores avanços têm ocorrido nos anos mais recentes. Atualmente a técnica de neurorrafia preferencial é a sutura epineural em detrimento das suturas interfasciculares, pelas razões expostas no capítulo anterior. Apesar de, quer na neurorrafia topo-a-topo quer no caso de interposição de enxertos, as técnicas atuais permitirem já alguns resultados positivos, alguma inovação é ainda possível, através do uso de colas cirúrgicas que apresentam alguns resultados promissores (Zorn, Bernstein et al. 2008, Whitlock, Kasukurthi et al. 2010, Sameem, Wood et al. 2011), ou ainda a cirurgia assistida por robôs (Liverneaux, Nectoux et al. 2009, Nectoux, Taleb et al. 2009) que apresentam igualmente alguns resultados encorajadores (Latif, Afthinos et al. 2008).

A utilização de técnicas de transplantação é aquela que maior interesse tem despertado dentro da medicina regenerativa e podem envolver transplante do órgão completo, de parte do órgão (transplante tecidular), de células (transplante celular) ou de constituintes sub-celulares (transferência genética) (Geuna, Gnavi et al. 2013).

No caso de lesões de nervo periférico, o **transplante de órgão ou tecido** é representado pelos autoenxertos (transplante de segmentos nervo autólogo) colhidos de outro nervo de menor importância que é assim sacrificado. Esta técnica foi realizada a primeira vez por Millesi (Millesi, Meissl et al. 1972), e atualmente representa ainda o G*old Standard* para resolução de lesões de nervo periférico em que a sutura topo-a-topo não é possível ou apresenta demasiada tensão (Siemionow and Brzezicki 2009). As principais desvantagens são a necessidade de sacrifício de outro nervo e o facto de nervos de menor calibre ou sensitivos serem utilizados com menor taxa de sucesso, entre outras descritas na secção 3 do capítulo I (Millesi H 1990, Madison RD 1992, Dahlin and Lundborg 1998, Flores AJ 2000, Lee AC 2003, Varejão. 2003, Udina E 2004, Bellamkonda 2006, Tyner, Parks et al. 2007), pelo que novas soluções têm sido procuradas nos últimos anos.

Algumas das soluções alternativas aos autoenxertos de origem biológica são a utilização de veias (Terzis and Karypidis 2009), técnica descrita pela primeira vez em 1909 (Wrede 1909) e a utilização de tecido muscular, descrito pela primeira vez em

1940 (Kraus and Reisner 1940) sendo que alguns autores obtiveram resultados efetivos em alguns estudos clínicos com ambos os tecidos (Mligiliche, Tabata et al. 2001, Meek M F 2002). Outros autores descreveram ainda uma combinação de ambos os tecidos para mitigar as limitações de cada um deles em separado (Brunelli and Brunelli 1993, Tos P 2004, Tos, Battiston et al. 2007). Uma outra hipótese é a utilização de aloenxertos, que parece ser uma técnica promissora e com resultados similares ao uso de autoenxertos (Brooks, Weber et al. 2012).

O **transplante celular** é atualmente uma área de investigação na regeneração de nervo periférico, sendo as células gliais as mais utilizadas, dado o seu papel preponderante na regeneração axonal (Geuna, Raimondo et al. 2009), formando as bandas de Büngner, segregando fatores de crescimento e juntamente com os macrófagos removendo o tecido necrótico e detritos de mielina (Hall 2001, Geuna, Raimondo et al. 2009), pelo que a sua presença ou dos seus percursores dentro de tubo-guia (naturais ou sintéticos), melhora significativamente a qualidade e taxa de regeneração (Hadlock, Sundback et al. 2000, Mosahebi, Woodward et al. 2001, Strauch, Rodriguez et al. 2001, Zhang, Blain et al. 2002, Goto, Mukozawa et al. 2010).

No entanto o seu emprego clínico levanta diversos problemas devido ao tempo necessário para expandir o número destas células em cultura assim como o risco de contaminação por fibroblastos (Mosahebi, Woodward et al. 2001, Moreno–Flores, Bradbury et al. 2006) sendo os precursores neuro-gliais uma possível alternativa (Bithell and Williams 2005), mas ainda com resultados controversos (Murakami T 2003, Heine, Conant et al. 2004, Amado, Simoes et al. 2008, Amado, Rodrigues et al. 2010). Outro tipo de células utilizadas neste tipo de terapia são as células do revestimento olfatório (*olfactory ensheathing cells* ou OECs) que têm apresentado resultados promissores quer na regeneração do nervo periférico quer em lesões da espinhal medula (Verdú, Navarro et al. 1999, Guntinas-Lichius, Angelov et al. 2001, Radtke, Akiyama et al. 2005, Dombrowski, Sasaki et al. 2006, Radtke and Vogt 2009).

O uso de células estaminais mesenquimatosas (MSCs) tem sido uma outra opção de transplante celular em nervo periférico, dado serem facilmente obtidas, purificadas e expandidas em cultura celular oferecendo um fonte potencialmente ilimitada de células (Geuna 2001, Tohill and Terenghi 2004, Caplan and Dennis 2006). Estas células apresentam como vantagem poder ser obtidas de diversos

tecidos como medula óssea, tecido adiposo, polpa dentária, cordão umbilical (Alhadlaq and Mao 2004), e têm a capacidade de diferenciação em diversas linhagens celulares incluindo neuronal e glial, melhorando a regeneração (Alhadlaq and Mao 2004, Kingham, Kalbermatten et al. 2007, Cho, Jang et al. 2010, Mantovani, Mahay et al. 2010). Uma outra vantagem deste tipo celular advém do facto de mesmo que estas células não se diferenciem, elas têm capacidade de alterar o microambiente no qual se inserem secretando localmente fatores de crescimento e desta forma melhorar os índices regenerativos através da modulação e coordenação da reacção inflamatória local e sistémica (Caplan and Dennis 2006, Ankrum and Karp 2010, Bonfield, Nolan Koloze et al. 2010, F Azari, Mathias et al. 2010, Joyce, Annett et al. 2010, Meyerrose, Olson et al. 2010).

A **terapia genética** tem sido aplicada à regeneração de nervo periférico, fornecendo localmente fatores neurotróficos, através de vetores virais, evitando os efeitos secundários resultantes da administração por via sistémica (Zacchigna and Giacca 2009).

O desenvolvimento de **biomateriais** para substituição de nervo periférico e fabrico de tubos-guia é atualmente uma das áreas de maior investigação na regeneração de nervo periférico.

### 1.1. Biomateriais

Na reconstrução de nervo periférico em lesões com afastamento dos topos nervosos podem ser utilizados diversos materiais, alguns ainda em fase de estudo. Estes biomateriais são classificados em dois grandes grupos:

- Naturais
- Sintéticos

Os sintéticos são subdivididos em outros dois grupos:

- Biodegradáveis
- Não biodegradáveis

A utilização de tubos-guia assume cada vez mais um maior protagonismo, pois apresenta como principais vantagens relativamente aos autoenxertos: evitar-se uma segunda cirurgia para se efetuar a colheita do nervo; e o tubo-guia apresenta menos

limitações em termos de calibre, ao contrário do autoenxerto que regra geral é de menor calibre que o nervo a intervencionar.

No processo de desenvolvimento e fabricação dos tubos-guia, existem algumas propriedades essenciais para a sua utilização em regeneração de nervo periférico (Hudson, Evans et al. 1999):

- Biocompatibilidade (Capacidade de inibir processos lesivos à regeneração nervosa, como fibrose, formação de neuromas, isquemia, aderências ou edema, não tóxico, não carcinogénico)
- Têm de ser esterilizáveis

Além destas características essenciais, outras devem ser tidas em consideração por forma a melhorar o desempenho de um tubo-guia (Huang and Huang 2006):

- Tem de ser possível a produção de tubos-guia com diferentes diâmetros e espessuras, por forma a dar resposta aos diferentes calibres de nervos potencialmente lesados
- Têm obrigatoriamente de manter a força de tensão durante todo o processo de regeneração
- Devem ser de fácil implantação por técnicas de microcirurgia, por forma a evitar excessiva manipulação do nervo lesado
- Devem ser preferencialmente biodegradáveis
- Devem ter a porosidade adequada
- Flexível
- Transparente
- Deve ser possível, preferencialmente, a incorporação de sistemas celulares de suporte
- Devem apresentar a capacidade de controlar a libertação de fatores neurotróficos
- Devem fornecer substrato para orientar o nervo, promovendo vascularização e um ambiente rico em fatores neurotróficos

- Devem preferencialmente apresentar uma condutividade elétrica, pois a criação de campos elétricos pode apresentar um potencial impacto na regeneração nervosa

Algo crucial para a obtenção de bons resultados, é a facilidade de implantação dos tubos-guia e para tal são fundamentais algumas características físicas do próprio biomaterial, como sendo a sua **transparência**, que permite uma visualização de todo o processo de implantação, a **flexibilidade**, importante para uma penetração facilitada da agulha do fio de sutura, a **resistência mecânica**, ou seja a capacidade de manter a sua forma, sem colapsar quer durante o procedimento cirúrgico de implantação quer durante todo o processo de regeneração.

Os tubos-guia **biodegradáveis** são preferenciais em detrimento dos não biodegradáveis, visto serem absorvidos pelo organismo, desaparecendo do local de implantação, reduzindo dessa forma o risco de infeção e a resposta inflamatória crónica, com eventual fibrose associada, e a compressão nervosa concomitante. Os tubos-guia, devem ser **semipermeáveis (porosos)** para permitir a incorporação de sistemas que criem e mantenham o microambiente favorável aos processos de regeneração e ao mesmo tempo permitir a eliminação dos produtos resultantes do metabolismo celular (Lundborg G 1982, Gibson KL 1991, Madison RD 1992, Meyer RS 1997, Steuer H 1999, Hundson TW 2000, Lee WP 2000, Heijke GCM 2001, Meek MF 2001).

Estas propriedades básicas nem sempre são possíveis de associar ao mesmo tempo num só biomaterial, pelo que há que definir algumas prioridades relativamente às que são essenciais e preferenciais por forma a obter-se o melhor biomaterial possível.

Diversos investigadores têm desenvolvido e testado diversas opções para solucionar as lesões de neurotmese em que não é possível a anastomose topo-a-topo, alguns dos quais são já soluções clínicas existentes e aprovadas no mercado, enquanto outras apenas se encontram em fase de investigação, como podemos ver na **tabela 1** (Schmidt and Leach 2003, Pfister, Gordon et al. 2011). Na **tabela 2** (Schlosshauer, Dreesmann et al. 2006) estão descritos biomateriais disponíveis no mercado para aplicação em regeneração de nervo periférico.

Apesar de todo o desenvolvimento tecnológico, a sutura clássica topo-a-topo apresenta ainda grandes vantagens sobre qualquer dos biomateriais atualmente

| Enxerto | Referência |
|---|---|
| ***Enxerto com tecido autólogo*** | |
| 1. Enxertos nervosos | Lundborg, 1998; Mackinnon e Dellon, 1988 |
| 2. Enxertos venosos | Chui, 1995; Chiu et al., 1982; Risitano et al., 2002 |
| 3. Enxertos musculares | Fawcett, 1986; Battiston et al., 2000; Meek et al. |
| 4. Bainhas epineurais | Karacaoglu et al., 2001 |
| 5. Enxertos tendinosos | Brandt et al., 199 |
| ***Enxertos acelulares não-autólogos*** | |
| 1. Imunosupressão com aloenxertos | Jablecki e Pielka, 2004; Sen et al., 2005 |
| 2. Aloenxertos acelulares e xenoenxertos | |
| Descelularização térmica | Ide et al., 1998; Frerichs et al. 2002 |
| Tratamento com radiação | Hiles, 1972; Marmor, 1964 |
| Descelularização química | Sondell et al., 1998; Sonell et al., 1999 |
| 3. Submucosa de intestino delgado | Hadlock et al., 2001 |
| 4. Membrana amniótica humana | Meek et al., 2001; Mligiliche et al., 2002 |
| ***Materiais naturais*** | |
| 1. ECM protein-based materials | |
| Fibronectina | Tong et a.,1994; Ahmed e Brown, 1999 |
| Laminina | Kauppila et al., 1993; Dubovy et al., 2001; Dubovy, 2004; Chen et all, 2007 |
| Colagénio | Yoshii et al., 2002; Navarro et al., 1996; Heijke et al., 2001; Mackinnon e Dellon, 1990b; Archibald et al. 1995; Tyner et al., 2007 |
| 2. Materiais baseados em Ácido Hialurónico | Seckel et al., 1995 |
| 3. Fibrina/fibrinogénio | Ahmed et al., 2000; Herbert et al., 1998 |
| 4. Alginato, agarose | Hashimoto et al., 2002; Balgude et al., 2001; |
| 5. Quitosano | Haipeng et al., 2000 Zhang et al., 2008; Hu et al., 2008 |
| ***Materiais sintéticos*** | |
| 1. Materiais sintéticos biodegradáveis | |
| Poli(ácido láctico) (PLA) | Evans et al., 2000; Evans et al., 2002 |
| Poli (ácido glicólico) | Dellon e Mackinnon, 1988b |
| Poli(láctico-co- ácido glicólico) (PLGA) | Molander et al., 1982 |
| Poli(caprolactona) (PCL) | den Dunnen et al., 2000; Valero-Cabre et al., 2001 |
| Poli(L-láctico-co-caprolactona) (PLL/CL) | Navarro et al., 1996 |
| Poli(uretano) | Soldani et al., 1919 |
| Poli(organo)fosfazeno | Nicoli et al., 2000 |
| Poli(3-hidroxibutirato) (PHB) | Young et al., 2002 |
| Poli(etileno glicol) | Lore et al. 1999; Borgens et al., 2002 |
| Poli(caprolactona-co-etil) etileno fosfato (PCLEEP) | Gilchrist et al., 1998; Chew et al., 2007 |
| Ácido Poli(glicólico) (PGA) | Hagiwara, 2002; Inada, 2005 |
| Glicolido trimetileno carbonato (GTMC) | Rosen, 1992 |
| Poli(tereftalato-etil fosfoester) (PPE) | Sinis et al., 2005 |
| Vidro biodegradável | Schmidt et al., 1997 |
| 2. Materiais eletricamente ativos | |
| Membranas piezoeletricas | |
| Condutores elétricos | Dahlin e Lundborg 2001; Lundborg et al., |
| 3. Materiais sintéticos não-biodegradáveis | 1982a; Navarro et al., 1996; Lundborg et al., 2004; Dahlin et al., 2001; Mackinnon e Dellon |
| Silicone | |
| Gore-Tex oo ePTFE | Wood et al., 2009 |
| Silicone ou Polifosfoester (PPE) | Xu et al., 2003 |
| Poli-2-hidroxiethil metacrilato-co-metil metacrilato (PHEMA-MMA) | Walter et al., 1993; Midha et al., 2003 |
| Poli(acrilonitrile metacrilato) (PAN-MA) | Garrity, 1955 |
| Poli(etileno) (PE), Poli(vinilo) (PV) e tubos de borracha | Kim et al., 2008 |
| Poli(etileno-co-acetato de vinilo) (PEVA) | Fine et al., 2002; Barras et al., 2002 |
| Poli(sulfona) | Yu, 2003; Dodla, 2008 |
| 4. Sintético (não especificado) | |

**Tabela 1 - (Schmidt and Leach 2003, Pfister, Gordon et al. 2011)**

utilizados (Heijke GCM 2001), mas estes, quando utilizados em lesões com afastamento dos topos nervosos de 5 mm e quando comparados com a aplicação de enxertos, apresentam resultados muito semelhantes, (Archibald JS 1995) sem apresentarem a morbilidade associada à colheita do enxerto e em alguns casos apresentaram ainda a vantagem de diminuírem a formação de neuromas em comparação com os autoenxertos (Tyner, Parks et al. 2007).

| Nome | Fabricante | Material |
|---|---|---|
| Neurotube® | Synovis | Poli(ácido)glicólico |
| Neurolac® | Polyganics | Poli-(DL-lático-ε-caprolactona) |
| SaluBridge® | SaluMedical | Hidrogel de PVA |
| NeuraGen® | Integra | Colagénio |
| Neuromatrix® | Collagen Matrix | Colagénio tipo I |
| Neuroflex® | Collagen Matrix | Colagénio tipo I |

**Tabela 2 - Biomateriais comercializados para aplicação em regeneração de nervo periférico (Schlosshauer, Dreesmann et al. 2006)**

A utilização dos tubos-guia apresenta ainda uma outra vantagem, em que muitos investigadores têm focado a sua investigação, que é o facto de ser possível manipular o microambiente no seu interior com diversas substâncias, células ou tecidos, como solução salina e gel de colagénio (Labrador, Butí et al. 1998, Ceballos, Navarro et al. 1999), fibrina (Williams and Varon 1985, Williams LR 1987), laminina (Madison, da Silva et al. 1985, Madison, Da Silva et al. 1988, Tong, Hirai et al. 1994), fibronectina (Bailey, Eichler et al. 1993), músculo fresco (Varejão ASP 2003), células de Schwann (Guenard, Kleitman et al. 1992, Levi, Sonntag et al. 1997), fatores tróficos (Rich, Alexander et al. 1989), células nervosas diferenciadas *in vitro* (Luís, Rodrigues et al. 2008) e células estaminais (Maurício, Gärtner et al. 2011, Gärtner, Pereira et al. 2013, Gartner, Pereira et al. 2014).

Como foi acima descrito, existe neste momento uma enorme diversidade de biomateriais com diferentes vantagens e desvantagens, mas regra geral pode afirmar-se que os materiais de origem natural apresentam como principal vantagem a sua biocompatibilidade, enquanto os materiais sintéticos são preferidos pelas suas propriedades físico-químicas (ritmo de degradação, porosidade, força mecânica, condutividade elétrica, dureza e consistência), podendo cada uma destas características ser manipulada e controlada consoante os objetivos propostos (Schmidt and Leach 2003, Bruns, Stark et al. 2007). Os biomateriais sintéticos são frequentemente não biodegradáveis ou não-absorvíveis, pelo que por vezes se torna

necessário a remoção posterior do biomaterial, para que este não promova estenose no local, com implicações negativas na função nervosa (Merle, Dellon et al. 1989). No entanto, dada a necessidade de não toxicidade nem de reação imunogénica, os materiais sintéticos reabsorvíveis são preferíveis aos materiais de origem natural (geralmente mais imunogénicos) (de Ruiter, Malessy et al. 2009). Os materiais reabsorvíveis cuja velocidade de reabsorção é demasiado lenta podem atrasar a regeneração por compressão no local. No extremo oposto encontram-se os biomateriais cuja velocidade de degradação é demasiado rápida, podendo desaparecer ainda antes de se completar o processo de regeneração (Den Dunnen, Van der Lei et al. 1995).

Outro fator relevante é a biocompatibilidade, ou seja, o próprio biomaterial tem de ser inócuo para os tecidos assim como os produtos resultantes da sua degradação. As principais características dos materiais que influenciam a resposta dos organismos ou tecidos que os recebem são (Williams 2008):

- Composição do material, micro ou nanoestrutura, morfologia, cristalinidade e cristalografia
- Constantes elásticas
- Teor de água e balanço hidrofóbico-hidrofílico
- Macro, micro e nano-porosidade
- Composição química de superfície, gradientes químicos, mobilidade molecular de superfície
- Topografia de superfície
- Energia de superfície
- Propriedades elétricas de superfície
- Parâmetros de corrosão, perfil de libertação e toxicidade de iões metálicos (materiais metálicos)
- Perfil de degradação, produtos da degradação e sua toxicidade (materiais poliméricos)
- Aditivos, catalisadores, contaminantes e sua toxicidade (materiais poliméricos)
- Perfil de dissolução/degradação, toxicidade dos produtos de degradação (materiais cerâmicos)
- Perfil de libertação de detritos de desgaste

Assim, a caracterização de um biomaterial deve incluir as seguintes avaliações: morfologia (SEM – microscopia eletrónica de varrimento), temperatura vítrea, Tg (DSC- calorimetria diferencial de varrimento), perda de massa/alteração de massa molecular (TGA – análise gravimétrica e GPC – cromatografia de exclusão molecular), intumescimento e análise de degradação (Ratner, Hoffman et al. 2004), condutividade (Massazumi 2000), identificação de grupos químicos (espectroscopia de Infravermelhos com transformadas de Fourier), análise da presença de fases cristalinas (difração de raios-X), ensaio de molhabilidade (medição dos ângulos de contacto com água), estimativa da carga da superfície (análise do potencial Zeta) e propriedades elásticas (DMA - análise mecânica dinâmica).

A crescente associação de sistemas celulares a biomateriais obriga a estudos relativos à adesividade destes sistemas celulares à superfície do biomaterial, assim como da citocompatibilidade e interferências entre o material e os sistemas celulares utilizados (Harley, Hastings et al. 2006).

O desenvolvimento da química macromolecular permitiu a descoberta do poli(álcool vinílico) (PVA) em 1915 por F. Klate. Desde a altura dessa descoberta muitas aplicações para o PVA foram descobertas e ainda se continuam a encontrar novas aplicações para esta substância (Saxena 2004).

O poli (álcool vinílico) (PVA) tem uma estrutura química simples com um grupo hidroxilo pendente. Na Figura 13 podemos ver o monómero do poli (álcool vinílico).

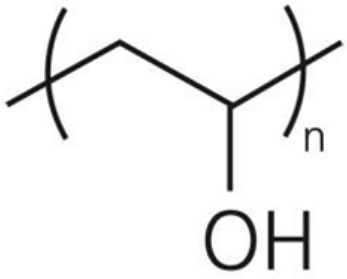



**Figura 13 - Unidade estrutura do poli (álcool vinílico).**

O PVA é produzido pela polimerização do acetato vinílico em acetato polivinílico (PVAc), seguido de hidrólise do acetato polivinílico em poli (álcool vinílico) (PVA). O poli (álcool vinílico) é um polímero hidrofílico e tem como vantagem ser insípido, inodoro, possuir excelentes propriedades mecânicas e ser biocompatível. As propriedades do PVA dependem do grau de polimerização e do grau de hidrólise. Este material pode ser comercializado como um copolímero hidrolisado, meio hidrolisado ou parcialmente hidrolisado, podendo também haver misturas

personalizadas (Hassan and Peppas 2000, Ogur 2005, Millon, Mohammadi et al. 2006, Reis, Campos et al. 2006, Alves, Jensen et al. 2011).

O PVA tem diversas aplicações devido à sua resistência contra solventes orgânicos e solubilidade aquosa. O PVA é utilizado na indústria têxtil, em embalagens de produtos alimentares e dispositivos médicos, com aprovação da FDA nos EUA. Nos dispositivos médicos, é utilizado como biomaterial dadas as suas características de biocompatibilidade (Alexandre, Ribeiro et al. 2014), turgidez (Chang 2010), bioadesividade, por ser não tóxico e não carcinogénico (Alves, Jensen et al. 2011, Baker, Walsh et al. 2012), sendo usado em lentes de contacto, como revestimento de corações artificiais, em aplicadores de administração de fármacos, como por exemplo como veículos de libertação de fármacos dadas as suas propriedades promotoras de uma interface hidrofílica e permeável (Kobayashi and Hyu 2010).

Este biomaterial mostra ser adequado para aplicação na produção de cateteres, em hemodiálise, membranas, na pele, cartilagem, músculo, e vasos sanguíneos, dado ser uma material suave, permeável e capaz de formar interfaces com os tecidos vivos, sendo indubitavelmente uma vantagem na área dos biomateriais e da indústria farmacêutica (Kobayashi and Hyu 2010). O PVA pode ainda ser associado a polímeros condutores elétricos que apresentam propriedades de tremenda importância, tornando-os capazes de substituir metais, fibras naturais, madeira ou outros materiais. O desenvolvimento de muitos destes materiais foi intensificado entre a primeira e a segunda grandes guerras mundiais. Em 1974 Hideki Shirakawa, com a descoberta do poliacetileno, provou que é possível obter polímeros condutores elétricos quase tão bons como os metais (Kungl 2000).

Os polímeros condutores atraíram a atenção de diversos grupos de investigação quer pela importância científica dos processos envolvidos quer pelo seu potencial em diversas aplicações tecnológicas. Estes novos materiais permitem a combinação de propriedades intrínsecas dos polímeros com propriedades elétricas, magnéticas e óticas dos metais e de semicondutores melhorando as propriedades e minimizando as deficiências (Henriques 2012).

A utilização de polímeros com condutividade elétrica como tubos-guia na regeneração nervosa despertaram bastante interesse pois apresentam simultaneamente propriedades físicas e químicas de polímeros orgânicos e características elétricas dos metais. Os polímeros condutores apresentam-se como

uma grande promessa na biomedicina nomeadamente na medicina regenerativa do nervo periférico. A importância destes materiais poliméricos é baseada na hipótese de que podem ser utilizados como matriz de suporte para crescimento de células, sendo possível a aplicação de estimulação elétrica a estas através do biomaterial, parecendo ter efeitos benéficos em muitas estratégias de regeneração (Al-Majed AA 2000a, Al-Majed AA 2000b, Brushart TM 2002, Brushart T M 2005, Chronakis, Grapenson et al. 2006, Willand, Nguyen et al. 2015).

O polipirrol (PPy) e os nanotubos de carbono (CNTs) são dois agentes utilizados na produção de polímeros condutores elétricos em engenharia. Outra solução é a incorporação de iões metálicos, particularmente quando o metal se liga quimicamente à cadeia polimérica, o que confere novas propriedades ao polímero ou melhora propriedades já presentes. Os iões de prata fornecidos pelo AgNO3 podem ser reduzidos a prata na superfície de filmes, obtendo-se filmes poliméricos metalizados condutores (Zidan 1999).

Os **nanotubos de carbono (CNTs)** foram descritos pela primeira vez por Oberlin et al. Em 1976, consistindo em folhas de grafeno enroladas com diâmetros na grandeza dos nanómetros e comprimentos na ordem de grandeza dos micrómetros. Os nanotubos estruturalmente pertencem à família do fulereno e são materiais com propriedade físicas, mecânicas e elétricas únicas que podem ser úteis em vários ramos da ciência dos materiais (Saito, Usui et al. 2009). Tipicamente, os CNT's são classificados como nanotubos de carbono de parede única (*single-walled carbon nanotube* – SWCNTs) ou de parede múltipla (*multiwalled carbon nanotubes* – MWCNTs). A estrutura dos SWCNTs pode ser conceptualizado como uma camada com a espessura de um átomo de grafite em forma cilíndrica sem qualquer tipo de emenda, enquanto os MWCNTs apresentam várias camadas de grafite. Diferentes estruturas manifestam também diferentes características, sendo que os SWCNTs exibem propriedades elétricas que os MWCNTs não apresentam, enquanto estes apresentam muito maior resistência a substâncias químicas que os primeiros (Dresselhaus, Dresselhaus et al. 1996) (Figura 14).

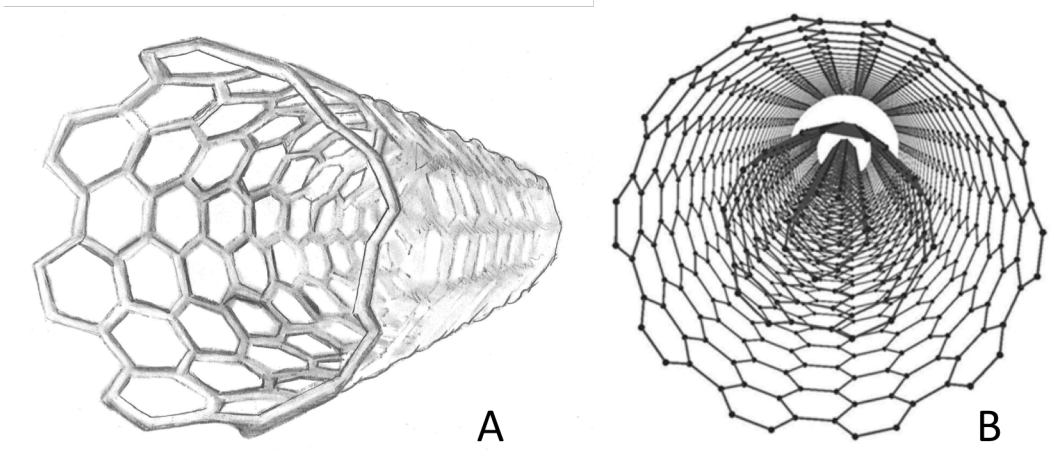



**Figura 14 – A -Estrutura tridimensional de Nanotubos de carbono (SWCNTs) (Ilustração de Duarte Monteiro); B - Estrutura tridimensional de Nanotubos de carbono (MWCNTs) Adaptado de (Sharma, Jelen et al. 2015)**

Uma das questões que se tem levantado e ainda hoje controversa, é a toxicidade associada a este tipo de biomateriais (Malarkey and Parpura 2007). No entanto, é já claro que este tipo de materiais será num futuro próximo muito útil em biomedicina (Lacerda, Bianco et al. 2006). Os CNTs são materiais resistentes, flexíveis, condutores de corrente elétrica e podem ser funcionalizados com diferentes moléculas, características que podem ser de extrema utilidade em biomateriais associados ao sistema nervoso (Saito, Usui et al. 2009). Os recentes desenvolvimentos tecnológicos melhoraram significativamente a biocompatibilidade, duração e robustez em substratos e aparelhos que afetam o crescimento neuronal e proporcionam terapias potenciais para desordens do SNC (Malarkey and Parpura 2007). Estudos recentes descobriram os efeitos que os CNTs exercem em células neuronais quando utilizados como substrato para o crescimento celular assim como a capacidade inerente de interferirem com a atividade elétrica neuronal (Sucapane, Cellot et al. 2009).

O **polipirrol (PPY)** é um composto químico formado por vários anéis pirrólicos, sendo um composto aromático heterocíclico e é um dos polímeros de nova geração (Harun, Saion et al. 2009) (Figura 15).

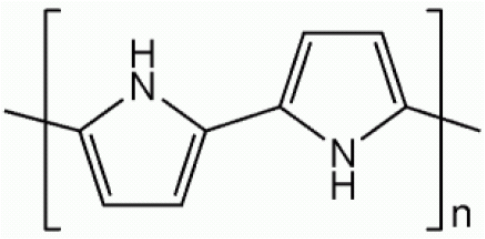


**Figura 15- Estrutura polimérica do PPY**

Este polímero sintético tem diversas aplicações em medicina, desde transporte e distribuição de fármacos, aplicação em regeneração nervosa, utilização em biossensores e como revestimento de sondas neurais. O elevado grau de conjugação da sua estrutura molecular central tornam-no muito rígido, insolúvel e difícil de processar (Ghasemi-Mobarakeh, Prabhakaran et al. 2011), sendo no entanto fácil de sintetizar, dado ser estável no meio ambiente e apresentando alta condutividade. Mostrou ainda ser citocompatível *in vitro* e *in vivo* (Williams and Doherty 1994, Chen, Wang et al. 2000, Wang, Gu et al. 2004, George, Saigal et al. 2009) e mostrou também suportar adesão celular de diversos tipos celulares e crescimentos destas, tornando-o um potencial candidato para a engenharia tecidular (Harun, Saion et al. 2009).

O **nitrato de prata** é um composto químico inorgânico com a fórmula $AgNO_3$, sendo uma substância com diversas utilizações ao nível químico, biológico, industrial e fundamentalmente em aplicações médicas, dadas as propriedades antissépticas dos sais de prata, ou como cauterizante (Hanif, Tasca et al. 2003, Silver, Phung et al. 2006). Mais recentemente o nitrato de prata foi associado ao PVA tendo sido estudadas as suas propriedades elétricas e ópticas. (Devi, Sharma et al. 2002).

O **cloreto de magnésio** é um composto químico com a fórmula $MgCl_2$, sendo um componente essencial em muitas reações enzimáticas, especialmente tendo em vista a produção de energia a nível celular, é de extrema importância para a manutenção da saúde do sistema nervoso. Pode ser usado para acalmar nervos hiperexcitáveis, sendo utilizado no tratamento de fadiga nervosa, tiques, espasmos, tremores, irritabilidade, hipersensibilidade, ansiedade, confusão, desorientação e arritmias cardíacas. Também o tratamento da doença de Parkinson passa pela suplementação com magnésio e a epilepsia é uma patologia em que os níveis de magnésio se encontram demasiado baixos no sangue, no líquido cefalorraquidiano e no cérebro, causando hiperexcitabilidade de algumas regiões cerebrais (Leaver, Parkinson et al. 1987, Holleman, Aylett et al. 2001, Hashimoto, Nishi et al. 2008, Eby

and Eby 2010). Este sal foi também utilizado para aumentar a condutibilidade elétrica em polímeros, nomeadamente no PVA (Di Noto and Vittadello 2002).

### 1.2. Terapias celulares

As células estaminais são atualmente consideradas como um elemento de grande potencial na medicina regenerativa em diversas áreas de investigação e em diferentes tecidos. Nesse sentido e dado a grande diversidade de opções nesta área específica de investigação foi necessário clarificar conceitos relativamente a estas células e às suas designações. As **células estaminais** (células-mãe ou células-tronco) são **células capazes de se auto-renovarem e de se diferenciarem em diferentes tipos de células ou tecidos:** são células indiferenciadas e não especializadas, têm a capacidade de se auto-renovar e dividir indefinidamente e são capazes de se diferenciar em linhagens celulares distintas.

Nem todas as células estaminais têm o mesmo potencial de diferenciação. As **células estaminais** podem ser obtidas a partir de (Dominici, Le Blanc et al. 2006):

1. **Embriões pré-implantação (células estaminais embrionárias)**
2. **Embriões pós-implantação e do feto (células estaminais fetais)**
3. **Placenta, ou de um organismo adulto (células estaminais adultas)**

Essa origem caracteriza as **3 grandes categorias de células estaminais** (Dominici, Le Blanc et al. 2006):

1. **Totipotentes** (têm um potencial de diferenciação ilimitado, podendo originar qualquer tipo de célula e tecido; existem no embrião pré-implantação com 3 dias de vida, estádio de 6-12 células);
2. **Pluripotentes** (têm um potencial de diferenciação mais limitado, podendo originar qualquer tipo de tecido do organismo, à excepção da placenta; existem no embrião pré-implantação com 6 dias de vida, o blastocisto, e no embrião pós-implantação);
3. **Multipotentes** (têm um potencial de diferenciação mais restrito, originando células de tecidos específicos; existem nos fetos tardios, no cordão umbilical, na placenta e nos tecidos adultos).

As células estaminais totipotentes são as primeiras células embrionárias, que resultam das primeiras divisões embrionárias e que têm capacidade de dar origem a um indivíduo completo. As células embrionárias pluripotentes são as células que existem no embrião no estádio de blastocisto e têm elevado potencial de diferenciação, podendo dar origem a todos os tipos celulares de um organismo adulto, menos a placenta (portanto não podem dar origem a um indivíduo independente). Por fim existem as células multipotentes, que têm uma menor capacidade de diferenciação. São designadas por células estaminais adultas ou somáticas. Existem nos tecidos adultos onde contribuem para a regeneração e renovação desses tecidos. Pensa-se que estas células pela menor capacidade de proliferação diferenciam-se apenas nos tecidos onde estão localizados. Atualmente, o grande problema das células estaminais adultas é que não existem bons marcadores moleculares que as permitam distinguir das outras células dos tecidos. As que se conseguem isolar com mais facilidade são as células estaminais hemopoiéticas (Dominici, Le Blanc et al. 2006).

A principal função das células estaminais adultas é manter e reparar os tecidos onde se encontram. Recentemente descobriu-se que existem mais tecidos com células estaminais do que inicialmente se pensava. É evidente que determinados tecidos apresentem mais células estaminais como a medula óssea, todos os epitélios respiratórios, gastrointestinal e pele, pela própria necessidade que têm de se regenerarem (Jackson, Majka et al. 2002).

Uma característica importante das células estaminais adultas é que estas têm maior plasticidade do que se pensava inicialmente, ou seja, têm capacidade de se diferenciar em 5 tipos celulares, quando dadas as condições ambientais necessárias para tal. Estas têm a capacidade de se diferenciarem em todos os tipos de células sanguíneas (denominadas células estaminais hematopoiéticas) podendo ser igualmente obtidas a partir da medula óssea, sangue periférico, cordão umbilical e polpa dentária (Gronthos, Brahim et al. 2002, Jackson, Majka et al. 2002, Horwitz, Le Blanc et al. 2005). Através de um processo de transdiferenciação têm a capacidade de dar origem a um tipo celular para o qual não estavam inicialmente programadas. Este é um processo interessante do ponto de vista da medicina regenerativa porque permite, a partir de células do mesmo indivíduo obter células capazes de se transdiferenciar e serem utilizadas no mesmo individuo de onde essas células foram

retiradas. As terapias do futuro visam manipular as células estaminais adultas para que estas se transformem noutro tecido que não aquele do qual foram extraídas.

Neste sentido, as células estaminais pluripotenciais ou multipotenciais, têm sido alvo de bastante interesse na regeneração tecidular (Salgado, Oliveira et al. 2013). Dentro das diferentes populações de células estaminais, as células estaminais mesenquimatosas (MSCs) têm-se evidenciado (Chen, Shao et al. 2008). De acordo com a *International Society for Cell Therapy (ISCT)* existem alguns critérios mínimos para a identificação de populações de MSCs (Horwitz, Le Blanc et al. 2005, Dominici, Le Blanc et al. 2006):

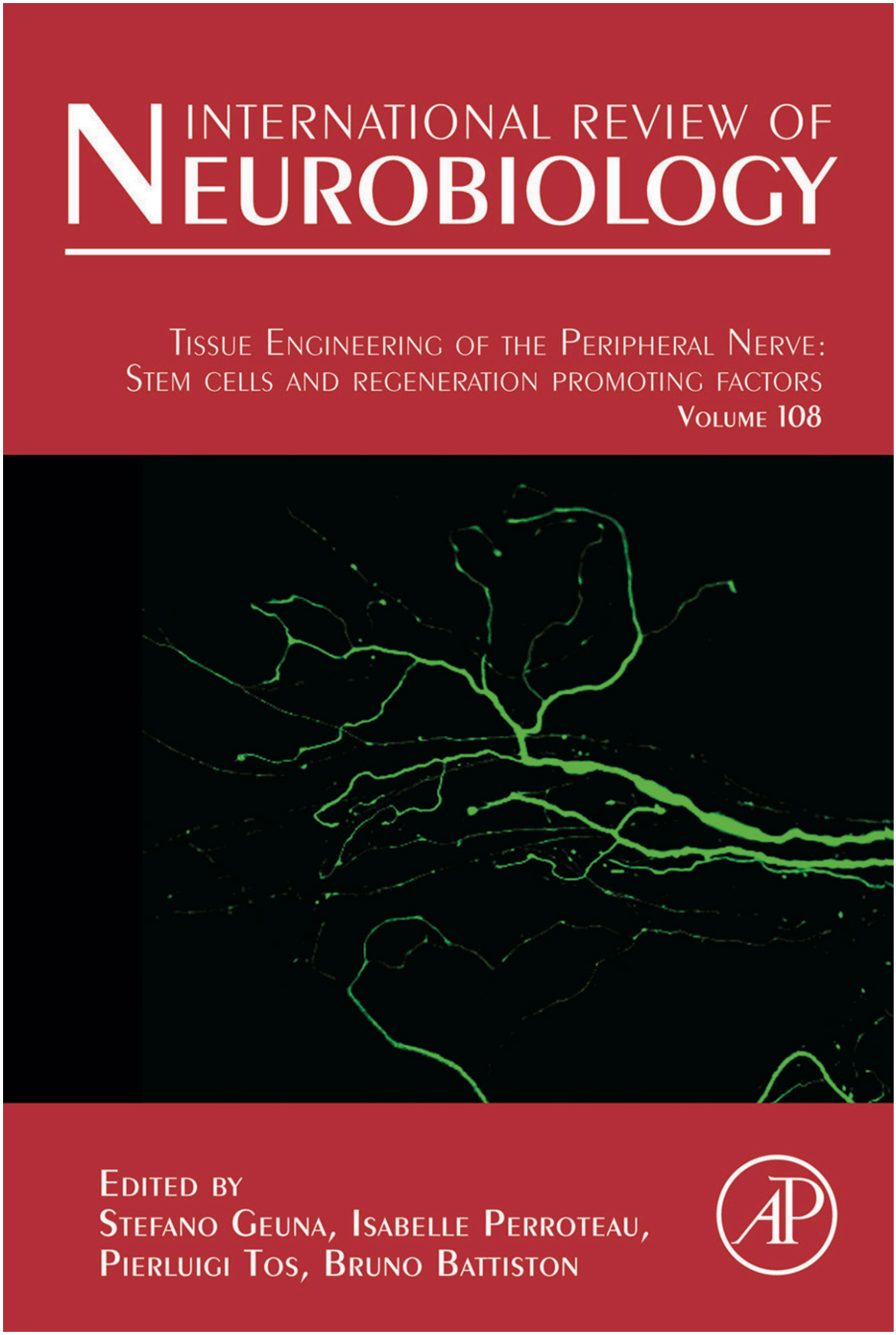
- Aderência ao plástico em condições de cultura *standard*
- Expressão positiva de marcadores específicos como os marcadores CD73, CD90 e CD105
- Expressão negativa de marcadores hematopoiéticos como os marcadores CD34, CD45, HLA-DR, CD14 ou CD11B, e CD79a ou CD19
- Diferenciação *in vitro* em pelo menos 3 linhas celulares diferentes nomeadamente osteogénica, condrogénica e adipogénica

A maioria das vezes, as células com este fenótipo podem ser isoladas a partir da medula óssea (BM-MSCs), tecido adiposo (ASCs) ou Geleia de Wharton do cordão umbilical (WJ-MSCs / HUCPVCs) (Salgado, Oliveira et al. 2006, Silva, Gimble et al. 2013, Teixeira, Carvalho et al. 2013).

Após a descoberta das células estaminais, tornou-se progressivamente claro que essas células poderiam ser utilizadas como um sistema natural de reparação tecidual (Phinney and Prockop 2007). Em vários estudos as células estaminais provaram ser um agente terapêutico eficaz em modelos experimentais de lesões dos tecidos (Minguell and Erices 2006). Consequentemente, a capacidade de reparação é secundária à secreção de fatores solúveis pelas células que alteram o microambiente do tecido. Nos anos mais recentes tem havido uma mudança no paradigma para o uso de MSCs na reparação e regeneração tecidular (Lai, Arslan et al. 2010, Baglio, Pegtel et al. 2012), pois estas células sobrevivem nos tecidos por diferentes períodos de tempo após transplantação. De facto, as propriedades imunomoduladoras dos secretomas de MSCs foi já demonstrado quer *in vitro* quer *in vivo* (Caplan and Dennis 2006, Ankrum and Karp 2010, Bonfield, Nolan Koloze et al. 2010, F Azari, Mathias et al. 2010, Joyce, Annett et al. 2010, Meyerrose, Olson et al.

2010). Assim, independente da capacidade de diferenciação destas células em células do tecido onde são aplicadas, é cada vez mais aceite que, por exemplo, ao nível da regeneração axonal existem diversos fatores neurotróficos com influência positiva nesta regeneração, como são NGF, NT-3, VEGF, GDNF e altos níveis de BDNF (Lu, Jones et al. 2005, Zhang, Zhang et al. 2009, Liu, Cheng et al. 2011, Lopatina, Kalinina et al. 2011). Além da produção dos fatores neurotróficos acima descritos e que têm efeito positivo na regeneração de nervo periférico, temos também de considerar a capacidade que estas células apresentam de se diferenciar em células tipo neuro-gliais (Shen, Duan et al. 2010, Cheng, Duan et al. 2011, Gartner, Pereira et al. 2012, Gärtner, Pereira et al. 2013). Em estudos recentes, foi demonstrado que BM-MSCs eram capazes de se diferenciar em células de Schwann capazes de suportar a regeneração de nervo periférico (Ladak, Olson et al. 2011) e inclusive foi verificado que exerciam efeitos neurotróficos semelhantes a células de Schwann normais (Ladak, Olson et al. 2011). Neste sentido, esta solução foi investigada nos trabalhos incluídos nesta dissertação, devido aos resultados promissores encontrados nos estudos mais recentes.

## 2. Perspectives of employing Mesenchymal Stem Cells from the Wharton's Jelly of the Umbilical Cord for Peripheral Nerve Repair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## Tissue Engineering of the Peripheral Nerve

### Stem Cells and Regeneration Promoting Factors


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### Stem Cells and Regeneration Promoting Factors

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# CONTENTS

CHAPTER FOUR

# Perspectives of Employing Mesenchymal Stem Cells from the Wharton's Jelly of the Umbilical Cord for Peripheral Nerve Repair

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## Contents



## Abstract

Mesenchymal stem cells (MSCs) from Wharton's jelly present high plasticity and low immunogenicity, turning them into a desirable form of cell therapy for the injured nervous system. Their isolation, expansion, and characterization have been performed from cryopreserved umbilical cord tissue. Great concern has been dedicated to the collection, preservation, and transport protocols of the umbilical cord after the parturition to the laboratory in order to obtain samples with higher number of viable MSCs without microbiological contamination. Different biomaterials like chitosan-silicate hybrid,

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collagen, PLGA90:10, poly(DL-lactide-ε-caprolactone), and poly(vinyl alcohol) loaded with electrical conductive materials, associated to MSCs have also been tested in the rat sciatic nerve in axonotmesis and neurotmesis lesions. The *in vitro* studies of the scaffolds included citocompatibility evaluation of the biomaterials used and cell characterization by imunocytochemistry, karyotype analysis, differentiation capacity into neuroglial-like cells, and flow cytometry. The regeneration process follow-up has been performed by functional analysis and the repaired nerves processed for stereological studies permitted the morphologic regeneration evaluation. The MSCs from Wharton's jelly delivered through tested biomaterials should be regarded a potentially valuable tool to improve clinical outcome especially after trauma to sensory nerves. In addition, these cells represent a noncontroversial source of primitive mesenchymal progenitor cells, which can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses. The importance of a longitudinal study concerning tissue engineering of the peripheral nerve, which includes a multidisciplinary team able to develop biomaterials associated to cell therapies, to perform preclinical trials concerning animal welfare and the appropriate animal model is here enhanced.

## 1. INTRODUCTION

Tissue engineering focusing on the *in vitro* fabrication of autologous, living tissues with the potential of regeneration is a promising scientific and clinical field. Peripheral nerve regeneration should include a multidisciplinary team able to develop biomaterials, to develop cell therapies, and to elaborate *in vitro* analysis and preclinical trials concerning animal welfare and the most appropriate animal model before the clinical trials and clinical application approval (Hermann et al., 2004). A full understanding of nerve regeneration, especially complete functional achievement and organ reinnervation after nerve injury, still remains the principal goal of regenerative medicine. The reliability of animal models is crucial for peripheral nerve research. Because of its peripheral nerve size, the rat sciatic nerve has been the most commonly used experimental model in studies concerning the peripheral nerve regeneration and possible therapeutic approaches (Kerns, Braverman, Mathew, Lucchinetti, & Ivankovich, 1991). Although sciatic nerve injuries themselves are rare in humans, this experimental model provides a very realistic testing bench for lesions involving plurifascicular mixed nerves with axons of different size and type competing to reach and reinnervate distal targets (Amado et al., 2008; Sporel-Ozakat, Edwards, Hepgul, Savas, & Gispen, 1991). Focal crush causes axonal interruption but preserves the connective sheaths (axonotmesis). After this injury,

regeneration is usually successful since axons regenerate at a steady rate along the distal nerve supported by the reactive Schwann cells (SCs) and by the preserved endoneural tubules, which enhance axonal elongation and facilitate adequate reinnervation (Nichols et al., 2005). Crush injuries are appropriate to investigate the cellular and molecular mechanisms of peripheral nerve regeneration and to assess the role of different factors in the regeneration process (Mackinnon, Hudson, & Hunter, 1985). Nerve crush injury is also a well-established model in experimental regeneration studies to investigate the impact of various pharmacological treatments (Amado et al., 2008; Chang, Auyang, Scholz, & Nichols, 2009; Pereira et al., 2006). This injury does not imply surgical reconstruction but due to the regeneration period needed, the neurogenic atrophy of the innervated muscles may occur, so, therapeutic approaches to successfully decrease this recovery time are also important. Among various types of peripheral nerve injuries, transection injuries where the nerve trunk is completely interrupted, especially those resulting in large neural gaps, may have a devastating impact on patients' quality of life, and in these cases reconstructive surgery is required as a therapeutic management to achieve nerve regeneration and function restoration (Gu, Ding, Yang, & Liu, 2011). Unlike the adult central nervous system that fails to spontaneously regenerate after injury, the peripheral nervous system (PNS) has an intrinsic regenerative ability to a certain extent. In response to small injuries, peripheral nerves can regenerate on their own over relatively short distances under appropriate conditions. After peripheral nerves are transected, a series of molecular and cellular events, collectively called Wallerian degeneration, are triggered throughout the distal stump of transected nerves and within a small zone distal to the proximal stump, resulting in the disintegration of axoplasmic microtubules and neurofilaments (Sabatier, To, Nicolini, & English, 2011). Within 24 h most axons along the distal stump of transected nerves are reduced to granular and amorphous debris; by 48 h the myelin sheath begins to get transformed toward the short segment (Joao, Amado, Veloso, Armada-da-Silva, & Mauricio, 2010). Then macrophages and monocytes migrate into the degenerating nerve stumps to remove myelin and axon debris, while SCs proliferate to form longitudinal cell columns, known as Bands of BÜngner (Luís, 2008). Under the influences of neurotrophic factors and extracellular matrix (ECM) molecules produced by SCs, the proximal portion of transected nerves sprouts new daughter axons to generate a "regenerating unit" that is surrounded by a common basal lamina (Dahlin, Johansson, Lindwall, & Kanje, 2009; Evans, 2001). New axonal sprouts usually emanate from the nodes of

Ranvier, and undergo remyelination by SCs. Functional reinnervation requires that the regenerating axons elongate under the mediation of growth cones until they reach their synaptic target, and in humans, axon regeneration occurs at a rate of about 2–5 mm/day; thus significant injuries may take many months to heal, so this delay is the cause of neurogenic muscular atrophy conducting to a poor functional recovery (Jiang, Lim, Mao, & Chew, 2010).

Tissue engineering of peripheral nerves associates biomaterials, like chitosan, poly(DL-lactide-ε-caprolactone) (PLC) copolyester, collagen, and other biomaterials, some of them, previously studied by our group (Amado et al., 2008; Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes, et al., 2008; Luria, Panasyuk, & Friedenstein, 1971) to cellular systems, able to differentiate into neuroglial-like cells or even by modulating the inflammatory process, which might improve nerve regeneration, in terms of motor and sensory recovery, and also, by shortening the healing period avoiding regional muscular atrophy. Cell transplantation has been proposed as a method of improving peripheral nerve regeneration (Chen et al., 2007). SCs, mesenchymal stem cells (MSCs), embryonic stem cells, and bone marrow stromal cells are the most studied support cell candidates. To implant cultured cells into defective nerves (with axonotmesis and neurotmesis injuries), there are two main techniques. The cellular system may be directly injected into the neural scaffold, which has been interposed between the proximal and distal nerve stumps or around the crush injury (in neurotmesis and axonotmesis injuries, respectively). It can also be performed by preadding the cells to the neural scaffold via injection or co-culture (in most of the cellular systems, it is allowed to form a monolayer) and then the biomaterial with the cellular system is implanted in the injured nerve (Luria et al., 1971).

## 2. REGENERATIVE MEDICINE AND PERIPHERAL NERVE INJURIES

### 2.1. Nerve reconstruction

Despite continuous refinement of microsurgery techniques, peripheral nerve repair still stands as one of the most challenging tasks in neurosurgery. Direct repair with end-to-end suture, should be the procedure of choice whenever tension-free suturing is possible; however, patients with loss of nerve tissue, resulting in a nerve gap, are considered for a nerve graft procedure (Luis et al., 2007). In these cases, the donor nerves used for grafting

Ranvier, and undergo remyelination by SCs. Functional reinnervation requires that the regenerating axons elongate under the mediation of growth cones until they reach their synaptic target, and in humans, axon regeneration occurs at a rate of about 2–5 mm/day; thus significant injuries may take many months to heal, so this delay is the cause of neurogenic muscular atrophy conducting to a poor functional recovery (Jiang, Lim, Mao, & Chew, 2010).

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are commonly expendable sensory nerves (Matsuyama, Mackay, & Midha, 2000). This technique, however, has some disadvantages, with the most prominent being donor site morbidity, which may lead to a secondary sensory deficit and occasionally neuroma and intensive pain. In addition, the donor and the recipient nerve diameters often do not match, which might be the basis for poor functional recovery (Matsuyama et al., 2000). Alternatives to peripheral nerve grafts include cadaver nerve segments allografts, end-to-side neurorrhaphy, and entubulation by means of autologous non-nervous tissues, such as vein and muscles (Walsh & Midha, 2009a, 2009b; Zheng & Cui, 2012) or natural and synthetic biomaterials recently revised by Gu et al. (2011). Just like most of tissue-engineered products, tissue-engineered nerve grafts are typically composed of a physical scaffold with the introduction of support cells and/or growth factors or other biomolecular components, which might improve the functional recovery after axonotmesis and neurotmesis injuries (Cheng et al., 2011; Luria et al., 1971; Shen et al., 2010; Zheng & Cui, 2010). So, entubulation for peripheral nerve repair is used for nerve defects that cannot be bridged without tension (Battiston, Geuna, Ferrero, & Tos, 2005). Nerves will regenerate from the proximal nerve stump toward the distal one, whereas neuroma formation and ingrowth of fibrous tissue into the nerve gap are prevented. Consequently, guidance of regenerating axons is not only achieved by a mechanical effect but also by a chemical effect (such as accumulation of neurotrophic factors) (Maurício et al., 2011). Nerve guides can be made of biological or synthetic materials and, among the latter, both nonabsorbable and biodegradable tubes have been developed and preclinically tested. Some of them are nowadays used in patients and are available in the national and international market (Schmidt & Leach, 2003). The aim of our research group for the past 10 years has been exploring the therapeutic value of human umbilical cord (UC) Wharton's jelly derived from MSCs both *in vitro* and *in vivo*, associated to several tube-guides of natural or absorbable synthetic biomaterials on a rat sciatic nerve axonotmesis experimental model.

### 2.1.1 Biomaterials

Biomaterials used in biomedical sciences are developing continuously, bringing indisputable benefits to the clinical and research field of knowledge. The use of three-dimensional materials in tissue regeneration is now an increasingly used approach that takes the name of guided tissue regeneration, in which the regeneration of peripheral nerve is a strong candidate. The biomaterials can be biological and synthetic and, among the latter, both

nonabsorbable and biodegradable have been used (Schmidt & Leach, 2003). The concept behind the use of biodegradable biomaterials is that no foreign body material will be left in the host after the device has accomplished its task. For nerve lesion applications, these biomaterials may be used in the form of membranes or tubes, which function not only as a vehicle for the cellular system and/or therapeutic molecules, but also promotes a mechanical protection at the site of the injury. These scaffolds can be mounted in the nerve injury site as tube-guides by the surgeon, facilitating the microsurgical technique in case of neurotmesis injuries (Maurício et al., 2011). An ideal scaffold for peripheral nerve reconstruction has to satisfy many biological and physicochemical requirements, among which biocompatibility, biodegradability, permeability, biochemical properties, and surface properties are the most important concerns during the development of these scaffolds (Gu et al., 2011). On the other hand, the perfect biomaterial used for the construction of these membranes and tube-guides should follow the production requirements in terms of length and wall width concerning the nerve defect, be capable of supporting the suture, and if possible, be transparent. These properties are not mandatory, but can substantially improve the material handling during the surgical procedure and the microsurgery technique (Seckel, 1990). Sufficient mechanical strength must be considered when designing the tube-guides, in order to avoid the collapse during implantation and the healing period. The tube-guide at the same time should also be flexible to allow bending without breaking, so, the balance between flexibility and hardness is an important issue to be considered during its fabrication (Harley, Hastings, Yannas, & Sannino, 2006). Another important factor is the biomaterial resorption process and its degradation rate. The resorption of a biomaterial should be adjusted to the regeneration process, which depends on its molecular weight, composition, crystal structure, and thermal history. When associating a biomaterial to a cellular system it is also important to determine properties such as hydrophobicity, surface charge, and surface rugosity to establish its ability to support adhesion and cell growth (Harley et al., 2006). Among several biomaterials, our research group focused its attention on some biodegradable ones like those made of poly(lactic-co-glycolic) acid (PLGA), made of a novel proportion (90:10) of the two polymers, poly(L-lactide): poly(glycolide) (PLGA90:10), of PLC copolyester (Vivosorb® and Neurolac®), hybrid chitosan, collagen type III (Amado et al., 2010, 2008; Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes, et al., 2008; Maurício et al., 2011; Simoes et al., 2010; Zheng & Cui, 2010) and more recently, the poly(vinyl alcohol)

(PVA) loaded with electrical conductive materials, such as carbon nanotubes (CNTs) and polypyrrole (PPy) to produce a conductive biomaterial with higher electrical conductivity than the polymer matrix (data unpublished, under analysis).

#### 2.1.1.1 Natural biomaterials

The natural biomaterials are characterized by their biocompatibility, biodegradability, low toxicity, and low cost. In peripheral nerve lesions, our research group has been testing mainly two natural biomaterials for the past 5 years—the collagen and the chitosan (Amado et al., 2010, 2008; Maurício et al., 2011; Simoes et al., 2010; Zheng & Cui, 2010). Collagen is an integral component of the ECM of the nerve and several studies have used the collagen in peripheral nerve reconstruction (Gartner et al., 2012). In our studies, the equine collagen type III (GentaFleece®, Baxter, Nuremberg, Germany) was used with positive results, in axonotmesis and in directly sutured neurotmesis lesions (Amado et al., 2010; Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes, et al., 2008). The equine type III collagen membranes wrapped around the nerve lesion (crush injury) were used in order to support an experimental cellular system (N1E-115 cells *in vitro* differentiated for 48 h in the presence of dimethyl sulfoxide (DMSO). These cells were able to secrete neurotrophic factors in the injury site without being in direct contact with regenerating axons (Amado et al., 2010, 2008; Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes, et al., 2008). There are many studies published where collagen has been used for manufacturing tube-guides for nerve regeneration. Some of them, such as Neuragen®, are commercially available for clinical application in patients (Tyner et al., 2007).

Chitin is a biopolymer of *N*-acetyl-D-glucosamine monomeric units and it has been used in a wide range of biomedical devices. It is the second most abundant polysaccharide found in nature, right after cellulose. Chitosan is a copolymer of D-glucosamine and *N*-acetyl-D-glucosamine and its molecular structure is very similar to laminin, fibronectin, and collagen. Therefore, like collagen, chitosan has favorable biological properties for the nerve regeneration and it is easier to process than chitin. Chitosan is quite fragile in its dry form, so it has to undergo chemical cross-linking or has to be used with other biomaterials before scaffold fabrication. The chitosan (high molecular weight, Aldrich®, USA) tested by our research group was dissolved in 0.25 M acetic acid aqueous solution to a concentration of 2% (w/v). To obtain chitosan-silicate hybrid type III membranes, GPTMS (Aldrich®,

USA) was added to the chitosan solution and stirred at room temperature (RT) for 1 h. The drying process for type III chitosan membrane was as follows: (i) the solutions were frozen for 24 h at −20 °C and then transferred to the freeze-dryer, where they were left for 12 h to become complete dry; (ii) the chitosan type III membranes were soaked in 0.25 N sodium hydroxide aqueous solution to neutralize the remaining acetic acid, washed well with distilled water, and freeze dried (Amado et al., 2008; Simoes et al., 2010). All membranes used for *in vivo* testing were sterilized with ethylene oxide gas, considered by some authors the most suitable method of sterilization for chitosan membranes (Gärtner et al., 2012). Prior to their use *in vivo*, membranes were kept for 1 week at RT to clear any ethylene oxide gas remnants (Amado et al., 2008; Simoes et al., 2010). Results of the *in vivo* study showed that type III chitosan improved both nerve fiber regeneration and functional recovery in axonotmesis and neurotmesis injuries. In fact, the main morphological predictors of nerve fiber regeneration (number of fibers, axon and fiber size, and myelin thickness) were significantly improved in the experimental group of rats where the sciatic nerve was reconstructed with chitosan III in both types of lesions (Amado et al., 2008; Simoes et al., 2010). The same positive results with chitosan III were also obtained with respect to the main predictors of functional recovery, namely withdrawal reflex latency (WRL), extensor postural thrust (EPT), sciatic functional index (SFI) and static evaluation (SSI) tests, and motion analysis of ankle joint (Amado et al., 2008; Simoes et al., 2010). Chitosan III was developed as a hybrid of chitosan by the addition of GPTMS. The addition of GPTMS improves the wettability of chitosan surfaces (Amado et al., 2008; Shirosaki et al., 2005), and therefore chitosan type III is expected to be more hydrophilic than the original chitosan (Amado et al., 2008; Shirosaki et al., 2005). Chitosan type III was developed to be more porous, with a larger surface-to-volume ratio but preserving mechanical strength and the ability to adapt to different shapes. Significant differences in water uptake between commonly used chitosan and our hybrid chitosan type III were previously reported as a consequence of the difference in the ability of the matrix to hold water (Amado et al., 2008). In fact, hybrid chitosan-based membranes may retain about two times as much biological fluid as chitosan (Amado et al., 2008). A synergistic effect of a more favorable porous microstructure and physicochemical properties (more wettable and higher water uptake level) of chitosan type III and the presence of silicon ions may be responsible for the good results in promoting posttraumatic nerve regeneration (Amado et al., 2008). The significant improvement of axonal regeneration obtained

in crushed sciatic nerves surrounded by chitosan type III membranes suggests that this material may not just work as a simple mechanical scaffold but may work instead as an inducer of nerve regeneration. The neuroregenerative property of chitosan type III might be explained by the action on SC proliferation, axon elongation, and myelination (Shirosaki et al., 2005; Yuan, Zhang, Yang, Wang, & Gu, 2004). We have also tested both *in vitro* and *in vivo*, two types of hybrid chitosan membranes with the addition of an experimental cellular system of N1E-115 cells *in vitro* differentiated (Amado et al., 2008; Simoes et al., 2010). The N1E-115 cells derived from mouse neuroblastomas (Amado et al., 2008; Simoes et al., 2010) can *in vitro* differentiate into neuroglial-like cells (Amano, Richelson, & Nirenberg, 1972; Meek & Coert, 2002) and were used as a cellular model for MSCs. Previous results obtained by our research group using these N1E-115 cells *in vitro* differentiated showed that there was no significant effect in promoting axon regeneration, and they can even exert negative effects on nerve fiber regeneration specially in the case of neurotmesis injuries (Maurício et al., 2011; Simoes et al., 2010). The presence of transplanted N1E-115 cells in nerve scaffolds competing for the local blood supply of nutrients and oxygen and by their space-occupying effect could have hindered the positive effect of local neurotrophic factor release leading to a negative outcome on nerve regeneration, even in the presence of hybrid chitosan membranes, with previously proved positive effect in promoting the nerve regeneration (Amado et al., 2008; Maurício et al., 2011; Simoes et al., 2010).

#### 2.1.1.2 Synthetic biomaterials

They are already available in the market tube-guides (Neurolac®) constituted by poly(DL-lactide-ε-caprolactone) for clinical application. They are 16 mm long, with an internal diameter of 2 mm and a 1.5-mm thick wall (purchased from Polyganics BV, Groningen, The Netherlands) (Luis et al., 2007; Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes, et al., 2008). Neurolac® is stiffer than other biomaterials like PLGA, PVA, and hybrid chitosan (type II and type III) (Amado et al., 2008; Simoes et al., 2010) due to the structural reinforcement of the ester bonds. Neurolac® is the only transparent device approved by FDA, which is an important characteristic for the surgeon as it facilitates the insertion of the nerve stumps across the nerve gap. However, on the other hand, it is not flexible, which might make the microsurgery technique difficult during its implantation (Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes,

et al., 2008). The biodegradation rate of PLGA, which is controlled by the monomer ratio, molecular weight, and crystallinity, can range from weeks to months (Battiston et al., 2005; Luis et al., 2007). PLGA 90:10 was developed by our research group and intensively studied; it was obtained from their cyclic dimers, DL-lactide, and glycolide. Nonwoven constructs were used to prepare 16 mm long tube-guides, with an internal diameter of 2.0 and 1.5 mm thick wall, in order to be applied in a 10-mm sciatic nerve gap. These fully synthetic nonwoven materials are extremely flexible and biologically safe, and after implantation, they are able to sustain the compressive forces related to body movement. They have also some degree of porosity to allow the influx of low molecular nutrients required for nerve regeneration. The nonwoven structure allowed the tube-guide to hold the suture without difficulties; however, greater care had to be taken in order to ensure its integrity. These tube-guides of PLGA are expected to degrade to lactic and glycolic acids through hydrolysis of the ester bonds (Luis et al., 2007; Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes, et al., 2008).

It was tested *in vivo* in the PLC polymer (Vivosorb®) membranes (purchased from Polyganics BV, Groningen, Netherlands), in rat sciatic nerve axonotmesis and neurotmesis injuries (Gartner et al., 2012; Rodrigues et al., 2005) PLC membranes are hydrophilic, thereby allowing the water uptake; this is essential for the nutrient control and other metabolite transportation to the surrounding healing tissue. A few weeks after implantation, the mechanical strength gradually decreases and there is a loss of molecular weight as a result of the hydrolysis process. Approximately in 24 months, PLC degrades into lactic acid and hydroxyl-caproic acid, both safely metabolized into water and carbon dioxide and/or excreted through the urinary tract. In contrast to other biodegradable polymers, PLC has the advantage over PLGA of not creating an acidic and potentially disturbing microenvironment, which is favorable to the surrounding tissue (Luis et al., 2007). Our PLC studies (Gartner et al., 2012) demonstrated that this biomaterial does not interfere negatively with the nerve regeneration process. In fact, the information on the effectiveness of PLC membranes and tube-guides for allowing nerve regeneration was already attested in previous published clinical trials (Jones & McGonagle, 2008).

The functional unit of the nervous system, the neuron, is an electrically excitable cell that processes and transmits information by electrical and chemical signaling, so electrical conductivity is one of the crucial characteristics for an ideal nerve guidance channel (Guillot, Gotherstrom, Chan,

Kurata, & Fisk, 2007). One of the biomaterials that has been recently tested by our research team is a polymer named poly(vinyl alcohol) (PVA), loaded with electrical conductive materials, such as CNTs and PPy to produce a conductive biomaterial with higher electrical conductivity than the polymer matrix. The PVA is a water-soluble synthetic polymer and is prepared via the hydrolysis (alcoholysis) of poly(vinyl acetate) (PVAc), in which the acetate groups are replaced by hydroxyls (Gotherstrom, Ringden, Westgren, Tammik, & Le Blanc, 2003). The catalyst is sodium hydroxide, the nature of the distribution of the residual acetyl groups in the partially hydrolysed PVA is determined by the choice of catalyst and, where solvents are used, by the nature of those solvents (Gotherstrom et al., 2003). PVA has many applications due to its resistance against organic solvents and aqueous solubility. It is used in textile industries, in the food packaging industry, and in medical devices. US Food and Drug Administration (FDA) approved PVA to be in close contact with food products. In medical devices, PVA is used as a biomaterial due to its biocompatibility, swelling properties, bioadhesive characteristics, and for being nontoxic and noncarcinogenic (Secco et al., 2008; Soland et al., 2012). It is used in contact lenses, in the coating of artificial hearts and drug administration devices. For instance, it is used in drug delivery vehicles due to the permeability and hydrophilic interface fostering (Soland et al., 2012). This material is also suitable for the production of catheters, haemodialysis, membranes, artificial skin, vascular prostheses, and wound dressing (Porada & Almeida-Porada, 2012). PVA may mimic the regulatory characteristics of natural ECMs and ECM-bound growth factors, both in clinical applications and in basic biology studies. For nerve guides, the polymers with electrical conductivity have attracted interest because they simultaneously display the physical and chemical properties of organic polymers and the electrical characteristics of metals. Conductive polymers show great promise in biomedicine, namely for regenerative medicine of peripheral nerve. The importance of those polymers is based on the hypothesis that such biomaterials can be used to host the growth of cells, and electrical stimulation can be applied directly to the cells, which proved to be beneficial in many regenerative strategies (Wood et al., 2012). PPy and CNTs are two of the most used agents to produce electrical conductive polymers for tissue engineering. Besides, the introduction of metal ions into a polymer, particularly when the metal is linked chemically into a polymer chain, often imparts new or improved properties to the polymer (Porada et al., 2011).

Our research group has been testing the application of PVA in producing a tube-guide for peripheral nerve reconstruction associated to PPy and

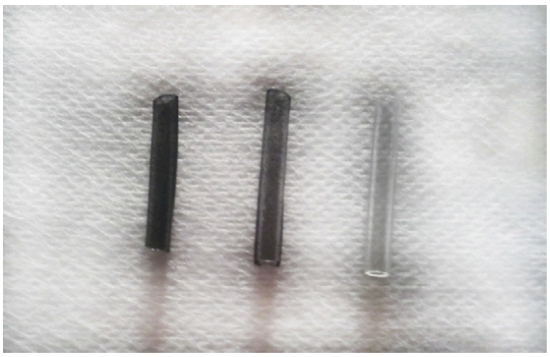

**Figure 4.1** Tube-guides used to reconstruct the rat sciatic nerve after axonotmesis and neurotmesis injuries. From the left to the right panel: PVA tube-guide loaded with CNTs, PVA tube-guide loaded with PPy and PVA tube-guide.

CNTs (Fig. 4.1). These tube-guides were already fabricated and implanted in the rat sciatic nerve after axonotmesis and neurotmesis injuries, and the nerve regeneration has been evaluated through functional tests described further ahead.

Synthetic biodegradable tubes of PVA (Aldrich, Mowiol 10-98) and PVA loaded with $COOH^-$-functionalized CNTs (Nanothinx, NTX5, MWCNTs 97% –COOH), and with PPy (Aldrich, $10^{-40}$ S/cm of conductivity), were prepared using a casting technique to a silicone mold. A 15% (%w/v) aqueous solution of PVA was prepared. Then the solution of PVA was mixed with 0.05% (%w/v) of $COOH^-$ functionalized and 0.05% (%w/v) of PPy. The tube-guides were produced with the following dimensions: length = 16 mm, $\text{diameter}_{in}$ = 2 mm, $\text{diameter}_{out}$ = 5 mm. The tube-guides were produced by a freezing/thawing process consisting in three cycles of freezer (−30 °C)/incubator (25 °C), and an annealing treatment started with a stage of 14 h on an incubator (25 °C) followed by a ramp rate of 0.1 °C/min until 80 °C, and then a stage of 20 h at 80 °C. Afterwards, the tube-guides were hydrated during 2 h before use. The electrical conductivity analysis of these prepared tube-guides showed a value of $1.5 \times 10^{-6}$ S/m for simple PVA tubes, which is according to bibliography (Park et al., 2001). The tubes with 0.05% (%w/v) of $COOH^-$-functionalized CNTs showed a value of $5.79 \times 10^{-4}$ S/m, whereas for PVA loaded with PPy they presented a value of $1.8375 \times 10^{-3}$ S/m. It is important to have in mind that PPy by itself has some conductivity (Fig. 4.1).

After obtaining these promising electrical conductivity values, the above two PVA nerve tube-guide compositions were chosen for further

characterizations and were applied in six groups of adult male Sasco Sprague–Dawley rats (Charles River Laboratories, Barcelona, Spain) with a standardized crush injury (axonotmesis) or a neurotmesis injury with a 10-mm gap. During the healing period, the regenerative process was evaluated by functional tests including kinematic analysis described further in this chapter. After the sacrifice of the experimental animals, 12 or 20 weeks postsurgery, with axonotmesis and neurotmesis injuries, respectively, the harvested regenerated nerves will be analyzed by histomorphometry (unpublished data).

Simultaneously, the biocompatibility of the PVA membranes was tested in subcutaneous implants in ovine (18 adult female white Merino sheep weighing approximately 60 kg) that were randomly divided in three groups of six animals each (one with PVA discs, one with PVA discs with a surface adsorbed with human MSCs derived from Wharton's jelly, and one control group). These PVA discs with 15.5 mm diameter were implanted subcutaneously. At 1, 2, 4, 8, 16, and 32 weeks postsurgery, the implants and the surrounding tissue were collected after a peripheral infiltration of 2% lidocaine and fixed with 10% formalin. Paraffin-embedded cuts of 2 μm were stained with hematoxylin and eosin (HE) for histological evaluation. The biological response parameters were assessed in the implant/tissue interface with three high power fields (×400) by at least two different pathologists for each sample and recorded in an appropriated formulary. Among the biological response parameters, all were evaluated according to the ISO standard 10993-6 (annex E) and included: the extent of fibrosis/fibrous capsule (layer in μm) and inflammation; the degeneration as determined by changes in tissue morphology; the number and distribution from the material/tissue interface of the inflammatory cell types, namely polymorphonuclear neutrophilic leucocytes (PMN), lymphocytes, plasma cells, eosinophils, macrophages, and multinucleated cells; the presence, extent, and type of necrosis; other tissue alterations such as vascularization, fatty infiltration, and granuloma formation; the material parameters such as fragmentation and/or debris presence, form, and location of remnants of degraded material and classified as nonirritant, slight irritant, moderate irritant, and severe irritant. According to this analysis, the PVA membranes alone or associated to the cellular system were considered slightly irritant ($N=24$ samples analyzed) (unpublished data).

### *2.1.2 Fetal-derived cells for tissue engineering*

Perinatal-derived cells are an ideal setting for tissue-engineering constructs as they can be harvested without harming intact donor structures and causing high risks for the child. The advantages for using these cellular systems are

the following: autologous cells source, processing with high quality standards and cryopreservation are possible, easy obtainable at various stages (pre and perinatal), present low immunogenic properties, these cell source often provides different types of cells including progenitor cells, the isolated cells have excellent cell growth capacities and they present a tumorogenic potential much lower when compared to omnipotent cells (Almeida-Porada, 2010; Cheng et al., 2011; Gartner et al., 2012; Hatlapatka et al., 2011; Maurício et al., 2011). In contrast to the highly standardized and industrially fabricated scaffolds, the quality of cells varies from patient to patient, depending on the individual tissue characteristics, on the transport conditions and time of the samples from the hospital/clinic to the laboratory, on the processing and cryopreservation techniques. These aspects are focused more ahead of this chapter concerning the collection of umbilical cord tissue (UCT) in order for isolation of MSCs from Wharton´s jelly (Cheng et al., 2011).

The MSCs isolated from extra-fetal tissues like the Wharton's jelly, the adipose tissue, and the bone marrow have a common morphologic description that includes a spindle shape, resembling fibroblasts, which are plastic adherent (Porada et al., 2010). The International Society of Cellular Therapy (ISCT) recognized the need to define the MSCs. This effort was made in order to distinguish between mesenchymal stromal cells and MSCs. The ISCT provides a clear and resumed definition: MSCs are progenitor cells for the mesenchymal lineages. These MSCs are adherent to tissue culture plastic (adherent cells), have a particular surface marker phenotype (express SH2, SH3, SH4, CD44, and HLA-class I, and do not express markers of the hematopoietic lineages CD34 and CD45 or HLA-class II), and have the capacity to differentiate into three mesenchymal lineages *in vitro* (bone, cartilage, adipocytes) (Dominici et al., 2006). The MSCs derived from the bone marrow and from the adipose tissue are able of self-renew and differentiate into specialized cells *in vitro*, there are reports of differentiation into neural cells (neurons, glial cells) (Cheng et al., 2011; da Silva et al., 2009; Gartner et al., 2012; Maurício et al., 2011).

UC-derived stem cells can be used for differentiation into all three layers, such as ekto-, endo-, and mesoderm (Cheng et al., 2011; Guillot et al., 2007; Hatlapatka et al., 2011). Concerning the nervous system, the UC-derived stem cells have been used for induction of neurons and glial cells (Cheng et al., 2011; da Silva et al., 2009; Gartner et al., 2012; Maurício et al., 2011). There are clear advantages of UC MSCs over the bone marrow MSCs. Obtaining bone marrow MSCs involves a painful bone marrow

aspirate, whereas the UC constitutes a waste product and a relatively high number of cells can be obtained by simple preparation of the cord. Extra-embryonic tissues are a good alternative to adult donor. These tissues, such as, amnion, microvillus, Wharton's jelly, and UC perivascular cells, are routinely discarded at childbirth, so slight ethical controversy attends the harvesting of the resident stem cell populations. The UC MSCs also represent "early" MSCs, which are considered superior cells obtained from more mature tissue like bone marrow, as they can undergo a significantly higher number of cell division before senescence (Cheng et al., 2011; Maurício et al., 2011). The reason for this higher proliferative capacity of fetal MSCs is because these cells have longer telomeres than adult MSCs (Galotto et al., 1999; Guillot et al., 2007). The fetal MSCs appear to lack some of the immune suppression properties observed in adult MSCs (Almeida-Porada, Zanjani, & Porada, 2010) and lack the human leukocyte antigen (HLA) class II, in contrast to adult MSCs and synthesize HLA-G, which is not present in the adult ones (Gotherstrom et al., 2003). Also, the cytokine profile of the fetal MSCs is different from that of the adult MSCs. So, the fetal MSCs like the ones isolated from the Wharton's jelly and from the UC blood, are primitive MSCs with greater ability to expand in culture due to their youth and naive status. MSCs have been isolated from several compartments of the UC, but our research group has been focused in the MSCs isolated from the Wharton's jelly and in preclinical studies regarding animal models of the application of this cellular system to the regeneration of the PNS after axonotmesis and neurotmesis injuries. Other important experimental work has been performed concerning the regeneration of bone, muscle, and vascular system concerning these MSCs but not referred since it is not included in the scope of this chapter (Shen et al., 2010).

## 2.2. MSCs from the Wharton jelly of the UC

### *2.2.1 Validation of transport of the UC from the hospital to the laboratory*

The comparatively large volume of extra-embryonic tissues increases the chance of isolating suitable amounts of MSCs, despite the complex and expensive procedures needed for their isolation. Some protocols use enzymatic digestion while others use enzyme-free tissue explant methods that require longer culture time (Gartner et al., 2013). Wharton's jelly is a mature mucous tissue and the main component of the UC, connecting the umbilical vessels to the amniotic epithelium. UC derives from extra-embryonic or embryonic mesoderm; at birth it weighs about 40 g and measures

approximately 30–65 cm in length and 1.5 cm in width (Gartner et al., 2013). Cord blood (CB) and more recently, UCT have been stored cryopreserved in private and public CB and tissue banks worldwide in order to obtain hematopoietic and MSCs and, although guidelines exist (Netcord—Foundation for the Accreditation of Cellular Therapy), standardized procedures for CB and UCT transport from the hospital/clinic to the laboratory, storage, processing, cryopreservation, and thawing are still awaited. These may be critical in order to obtain higher viable stem cells number after thawing and limit microbiological contamination (Cheng et al., 2011).

Our research group focused on determining whether UCT storage and transport from the hospital/clinic to the laboratory at RT or refrigerated (0.5–20 °C) and immersed in several sterile saline solutions affects the UCT integrity in order to be cryopreserved. Twelve UCs ($N=12$) were collected from healthy donors after written informed consent and following validated procedures according to the clinical and technical guidelines of the Portuguese Private Bank, Biosckin, Molecular and Cell Therapies, SA (authorized for processing and cryopreservation CB and UCT units by the Portuguese Authority Instituto Português de Sangue e Transplantação—IPST, IP). After collection in the hospital/clinic, these UCs were transported to the laboratory during 96 h at refrigerated temperature controlled by a datalloger. From each donor UC ($N=12$), eight fragments of 1 cm were cut, and each fragment from each different donor was immersed for 168 h in four different sterile saline solutions at RT (22–24 °C) and refrigerated (0.5–20 °C): NaCl 0.9% (Labesfal, Portugal), AOSEPT®-PLUS (Ciba Vision, Portugal), Dulbecco's phosphate-buffered saline without calcium, magnesium, and phenol red (DPBS, Gibco, Invitrogen, Portugal) and Hank's balanced salt solution (HBSS, Gibco, Invitrogen, Portugal). Two UCs ($N=2$) were immersed in 4% of paraformaldehyde and processed for light microscopy, one immediately after birth and other after 96 h at transport refrigerated temperature without being immersed in any of the tested sterile saline solutions. These two UCs served as control. After 168 h, the immersed fragments were collected in 4% of paraformaldehyde and processed for light microscopy. The samples were fixed in 4% paraformaldehyde for 4 h and then washed and conserved in phosphate buffer saline (PBS) until embedding. The specimens were dehydrated and embedded in paraffin and cut at 10 μm perpendicular to the main UC axis. For light microscope analysis, sections were stained with HE and observed with a Leica DM400 microscope equipped with a Leica DFC320 digital camera. The UCT

integrity was evaluated through the following parameters: (i) detachment of vessels and retraction of vascular structures; (ii) loss of detail and integrity of the endothelium; (iii) connective tissue degradation; and (iv) loss of detail and integrity of the mesothelium. It was concluded that the best transport solutions were HBSS or DPBS at refrigerated temperature, since those solutions maintained the histological structure of UC evaluated through those five parameters previously referred (Fig. 4.2). As a matter of fact, the UC immersed for 168 h in DPBS and HBSS at refrigerated temperature presented integrity of the histological structure comparable to UCs collected and processed for histological analysis immediately after birth and transported for 96 h after collection at refrigerated temperatures of transport. With DPBS, a slight retraction of the vessels was noted, which is advantageous since the vessels are stripped and discarded before cryopreservation of the UCT. It was concluded that the transport of the UC from the hospital/clinic to the cryopreservation laboratory when performed with the UC immersed in DPBS or HBSS at refrigerated temperatures, permits to extend the time of transport more than 96 h, which is the maximal time allowed according to the technical protocols of the Private Cord Blood Bank.

### 2.2.2 *Validation of the isolation protocol of MSCs from the Wharton's jelly*

Various methods of isolation have been described to obtain and isolate MSCs from the UC (Can & Karahuseyinoglu, 2007). Frequently, the

**Figure 4.2** Cross section of an umbilical cord transported immersed in DPBS at the refrigerated temperature 2 of 4–6 °C. Samples were stained with hematoxylin and eosin (HE). Magnification: 10×.

isolated MSCs although showing the properties and characteristics of MSCs, are probably different cell types. Most of the recently described methods share a common step, which is the manual separation of the tissue of interest within the UC, followed or not by enzymatic digestion of the ECM to release viable individual cells that are able to expand in culture.

Whether obtained through cesarean section or vaginal delivery, it is important to remove contaminating blood from the UC. As it was previously reported; CB itself can be a source of MSCs, but the rate of recovery is generally too low (Almeida-Porada et al., 2007). The blood vessels of the UC are another important source of MSCs (Romanov, Svintsitskaya, & Smirnov, 2003). It has been described in the isolation of MSCs from UC stripped vessels (Sarugaser, Lickorish, Baksh, Hosseini, & Davies, 2005) that these perivascular cells are isolated with collagenase incubation and present the MSCs characteristics like plastic adherence, surface markers expression, and ability to trans-differentiate in the presence of appropriate culture medium components. On the other hand, these perivascular cells express 3G5 (Sarugaser et al., 2005) and CD146 (Baksh, Yao, & Tuan, 2007), these two markers are not present in the MSCs from the Wharton's jelly. Wang and collaborators described the isolation of MSCs from the Wharton's jelly, after removing the UC vessels, by an enzymatic sequence of collagenase and trypsin (Wang et al., 2004). Weiss and collaborators also described an enzymatic method for the isolation of MSCs from the Wharton's jelly. After removing the UC vessels, the cord is cut in segments that are incubated with a cocktail of hyaluronidase, collagenase, and trypsin (Weiss et al., 2006). Other laboratories have been trying with success the isolation of MSCs from the Wharton´s jelly without enzymatic treatment like the published work by Mitchell et al. (2003). In this protocol, the cord tissue, after removal of the cord vessels, is cut in small segments that are placed directly in the culture dish (Mitchell et al., 2003).

The isolation and culture of MSCs from the Wharton's jelly was performed by our research group in order to obtain undifferentiated MSCs and *in vitro* differentiated neuroglial-like cells. Both cellular systems were tested *in vivo*, in axonotmesis and neurotmesis injuries of the rat sciatic nerve. The isolation was performed by enzyme-free tissue explant and by enzymatic isolation procedure. Despite our standard approaches, we are aware that there are still significant variations that exist between laboratories' protocols, which must be taken into account when comparing results using other methodologies. There is a wide range of individual differences among donor tissues also and our protocols usually use 15–20 cm of UC. While

most UC samples will provide a reasonable number of MSCs using the provided protocols, some samples may result in suboptimal cell isolation and expansion. The reasons behind this phenomenon still remain to be clarified, but as we have previously mentioned, the temperature and the time of transport from the hospital/clinic to the cryopreservation laboratory is crucial. Anyway, there is an important difference, described by Weiss et al., in 2006, between UC blood MSCs and Wharton's jelly MSCs: the latter can be isolated from close to 100% of the samples, even UCs that are delayed in their processing up to 48 hours (Weiss et al., 2006). These results were confirmed by our research group as it is described and discussed above.

Irrespective of the specific protocol, the washing procedure of the UC fragments is crucial in order to avoid microbiological contamination of the cultures. After obtaining the written informed consent from the parents, fresh human UCs are obtained after birth and transported from the hospital/clinic to the laboratory at refrigerated temperatures, as it was previously described. After washing the UC unit four times in rising sterile DPBS solution, disinfection is performed in 70% ethanol for 30 s. Finally, and before the dissection step, the UC unit is washed once again in sterile DPBS solution. The vessels are usually stripped with UC unit still immersed in DPBS. Once the washing step of the UC was considered essential to achieve good UCT units for cryopreservation and for MSCs isolation and culture, the washing protocol was validated. DPBS from the first washing step (used immediately after the transport of the unit to the laboratory—washing solution 1) and DPBS used in washing step after disinfection in 70% ethanol (washing solution 2) from 14 UC units ($N=14$) collected from healthy donors and transported from the hospital/clinical at refrigerated temperatures in <96 h, were tested for microbiological contamination using BacT/ALERT® (bioMérieux). Each unit was tested for aerobic and anaerobic microorganisms and fungi using 10 ml of the solution 1 and 10 ml of the solution 2, which were aseptically introduced into the BacT/ALERT® testing flasks. All procedures were performed in a laminar flow tissue culture hood under sterile conditions. All the units that presented microbial contamination in DPBS obtained from the first washing step (solution 1) presented no contamination in the analysis performed to DPBS from the last washing step immediately performed before MSCs isolation or UCT cryopreservation (solution 2). The following microorganisms were identified in the DPBS solution from solution 1: *Staphylococcus lugdunensis* ($N=2$); *Staphylococcus epidermidis* ($N=1$); *Staphylococcus coagulase* ($N=2$); *Escherichia coli* ($N=4$); *Enterococcus faecalis* ($N=1$); and *Streptococcus sanguinis* ($N=1$). The DPBS solution from

the first washing step (solution 1) from three UC units was negative for microbial contamination ($N=3$). These results permitted us to conclude that the washing protocol was 100% efficient in what concerns microbiological elimination (including aerobic and anaerobic bacteria, yeast and fungi) and appropriate for cryopreservation of UCT or for isolation and culture of MSCs obtained from the collected UCs (Cheng et al., 2011).

Once the transport and washing protocols were validated, it was important to isolate and expand *in vitro* the MSCs from the UCT units for future preclinical trials.

In the "enzymatic protocol," collagenase type I (Sigma-Aldrich) and trypsin-EDTA solution (Sigma-Aldrich) were used. With the written informed consent from the mother, fresh human UCs were obtained after birth and transported to the laboratory at refrigerated temperatures (Gibco, Invitrogen, Portugal) for 1–96 h before tissue processing to obtain MSCs. After removal of blood vessels, the Wharton's jelly is scraped off with a scalpel and centrifuged at 250 g for 5 min at RT and the pellet is washed with serum-free Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, Portugal). Next, the cells are centrifuged at $250 \times g$ for 5 min at RT and then treated with collagenase (2 mg/ml) for 16 h at 37 °C, washed and treated with 2.5% trypsin-EDTA solution (Sigma-Aldrich) for 30 min at 37 °C with gentle agitation. Finally, the cells are washed and cultured in DMEM (Gibco, Invitrogen, Portugal) supplemented with 10% fetal bovine serum (FBS), glucose (4.5 g/l), 1% (w/v) penicillin and streptomycin (Sigma-Aldrich), and 2.5 mg/ml amphotericin B (Sigma-Aldrich) in 5% $CO_2$ in a 37 °C incubator (Nuaire). Around $2 \times 10^5$ cells are plated into each T75 flask in 10 ml culture medium. Cells are allowed to attach and grow for 3 days. To remove the nonadherent cells or fragments, the flasks are gently washed using prewarmed DPBS after which 10 ml of prewarmed culture medium is added. The culture medium is changed every third day (or twice per week). Confluence (80–90%) is normally reached at day 12–16, and the cells are removed with prewarmed trypsin-EDTA solution (4 ml per flask), for 10 min at 37 °C. The cells are plated onto poly-L-lysine-coated glass coverslips (in 6- or 24-well tissue culture plates) (Sigma-Aldrich) or on biomaterials membranes used in the nerve reconstruction. Normally, 5000 cells/$cm^2$ are plated on the coverslips or on the membranes (Cheng et al., 2011) (Fig. 4.3).

In our "enzyme-free tissue explant protocol" for isolation of MSCs from the Wharton's jelly, enzymatic digestion is not employed. The mesenchymal tissue (Wharton's jelly) is diced into cubes of about 0.5 $cm^3$ and the

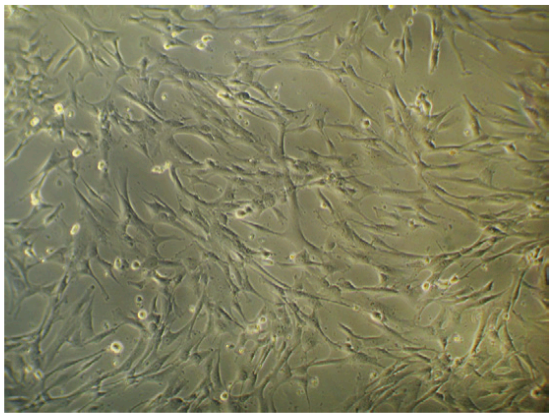

**Figure 4.3** MSCs isolated from Wharton's jelly using the "enzymatic protocol" exhibiting a mesenchymal-like shape with a flat polygonal morphology. Magnification: 100×.

remaining vessels are removed by dissection. Using a sterile scalp, the cubes are diced in 1–2 mm fragments and transferred to a Petri dish precoated with poly-L-lysine (Sigma-Aldrich) with mesenchymal stem cell medium (PromoCell, C-28010) supplemented with 1% (w/v) penicillin and streptomycin (Sigma-Aldrich), and 2.5 mg/ml amphotericin B (Sigma-Aldrich) and cultured in 5% $CO_2$ in a 37 °C incubator (Nuaire). Some tissue fragments will allow cell migration from the explants in 3–4 days incubation. Confluence is normally obtained 15–21 days after (Cheng et al., 2011) (Fig. 4.4).

The laboratory's processing and cryopreservation protocols of the UCT units following the technical procedures of Biosckin, Molecular and Cell Therapies S.A. (BSK.LCV.PT.7) were validated for the ability of isolating and expanding *in vitro* MSCs after cryopreserved UCT thawing. The protocols of processing and cryopreservation of the UCT are protected by a Confidentiality Agreement between Biosckin, Molecular and Cell Therapies S.A. and all the involved researchers. Briefly, the UCT collected from healthy donors ($N=60$), and according to Netcord guidelines and following the Portuguese law 12/2009 (Diário da República, lei 12/2009 de 26 de Março de 2009), was diced into cubes of about 0.5 $cm^3$ and the remaining vessels were removed by dissection. In order to ensure the viability of the UCT after parturition and limit the microbiological contamination of the samples, the UCs were transported from the hospital/clinic to the laboratory at refrigerated temperatures monitored by a datalloger in less than 96 hours. The UCT units from 15 to 20 cm long UCs and after the blood vessels

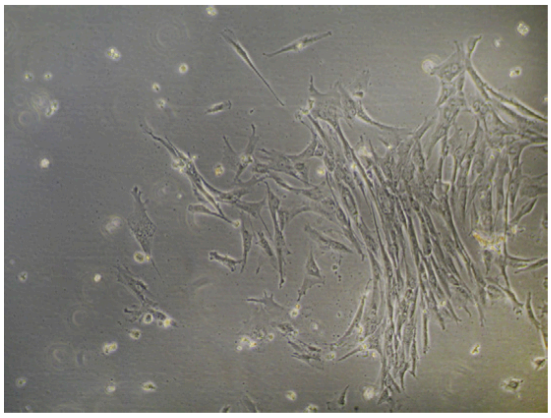

**Figure 4.4** MSCs isolated from Wharton's jelly using the "enzyme-free tissue explant protocol" exhibiting a mesenchymal-like shape with a flat polygonal morphology. The mesenchymal tissue (Wharton's jelly) 1–2 mm fragments transferred to a Petri dish precoated with poly-L-lysine allowed cell migration from the explants in 3–4 days incubation.

dissection were treated and processed for cryopreservation using a cryoprotective solution (freezing medium). The UCT units were transferred to a computer-controlled slow rate freezer (Sylab, Consensus, Portugal) and a nine-step freezing program was used to set up the time, temperature, and rates specifically optimized for the human UC-MSCs cooling. To thaw frozen cells, the cryovials were transferred directly to a 37 °C water bath. Upon thawing in less than a minute, the cell suspension was centrifuged at 250 × *g* for 10 min, and the supernatant was gently removed and the cell pellet was resuspended in culture medium (Cheng et al., 2011). It was possible to obtain MSCs in culture from 52 out of 60 thawed UCT units. In some UCT cryopreserved units ($N=8$), it was not possible to isolate MSCs due to increased number of erythrocytes' lysis and/or microbiological contamination during the initial cell culture period. The MSCs morphology was observed in an inverted microscope (Zeiss, Germany) at different points of expansion. The MSCs exhibited a mesenchymal-like shape with a flat and polygonal morphology. The MSCs obtained ($N=52$) were characterized by flow cytometry (FACSCalibur®, BD Biosciences) analysis for a comprehensive panel of markers, such as PECAM (CD31), HCAM (CD44), CD45, and Endoglin (CD105). The karyotype of undifferentiated MSCs was determined and no structural alterations were found demonstrating absence of neoplasic characteristics in these cells, as well as chromosomal stability to

the cell culture procedures. In the presence of neurogenic medium, the MSCs were able to become exceedingly long and there was a formation of typical neuroglial-like cells with multibranches and secondary branches. These results permitted to conclude that the processing and cooling protocols used for UCT units' cryopreservation were adequate to preserve the UCT viability since it was possible to isolate and expand MSCs after appropriate thaw and in presence of adequate cell culture conditions (Cheng et al., 2011).

### 2.2.3 MSCs cells from a cell line and differentiation into neuroglial-like cells

An established and ready-to-use human MSC cell line from the UC matrix (Wharton's jelly) was employed for promoting axonotmesis and neurotmesis lesions regeneration in the rat sciatic nerve model. Human MSCs from Wharton's jelly UC were purchased from PromoCell GmbH (C-12971, lot-number: 8082606.7). Cryopreserved cells were cultured and maintained in a humidified atmosphere with 5% $CO_2$ at 37 °C. Mesenchymal Stem Cell Medium (PromoCell, C-28010) was replaced every 48 h. At 80–90% confluence, cells were harvested with 0.25% trypsin with EDTA (Gibco) and passed into a new flask for further expansion. MSCs at a concentration of 2500 cells/ml were cultured on poly-D-lysine coverslips (Sigma-Aldrich) or on biomaterials membranes and after 24 h cells exhibited 30–40% confluence. Differentiation into neuroglial-like cells was induced with MSC neurogenic medium (PromoCell, C-28015). Medium was normally replaced every 24 h during 3 days. The formation of neuroglial-like cells was observed after 24 h in an inverted microscope (Zeiss, Germany) (Cheng et al., 2011; Gartner et al., 2012; Gärtner et al., 2012; Shen et al., 2010).

This established human MSC cell line was preferred for *in vivo* testing in rats, since the number of MSCs obtained was higher in a shorter culture time, it was not dependent on donors' availability and ethic committee authorization, and the protocol was much less time-consuming, which was advantageous for preclinical trials with a large number of experimental animals. As a matter of fact, there was no need of administrating immunosuppressive treatment to the experimental animals during the entire healing period after the surgical procedure. The phenotype of MSCs was assessed by PromoCell. Rigid quality control tests are normally performed for each lot of PromoCell MSCs isolated from Wharton's jelly of UC. MSCs are tested for cell morphology, adherence rate, and viability. Furthermore, each cell lot is characterized by flow cytometry analysis for a comprehensive panel of markers (Gartner et al., 2012).

The MSCs established PromoCell cell line exhibited a mesenchymal-like shape with a flat and polygonal morphology. During expansion, the cells became long spindle shaped and colonized the whole culturing surface. After 96 h of culture in neurogenic medium, cells changed in morphology. The cells became exceedingly long and there was a formation of typical neuroglial-like cells with multibranches and secondary branches (Cheng et al., 2011; Gartner et al., 2012; Gärtner et al., 2012). Giemsa-stained cells of *in vitro* differentiated MSC cell line at passage 5 were analyzed for cytogenetic characterization. The karyotype of undifferentiated MSCs obtained from the UCT cryopreserved and isolated by the enzyme-free tissue explants ($N=52$) (described in Section 2.2) and from PromoCell was determined and no structural alterations were found demonstrating absence of neoplasic characteristics in the MSCs cells, as well as chromosomal stability to the cell culture procedures (Gartner et al., 2013). The *in vitro* differentiated MSCs karyotype could not be established, since no dividing cells were obtained at passage 5, which can be in agreement with the degree of differentiation. The karyotype analysis of undifferentiated MSCs previously determined, supported the suitability of our cell culture and differentiation protocols. This concern resulted from previous negative *in vivo* results obtained with *in vitro* differentiated N1E-115 cells in axonotmesis and neurotmesis injuries of the rat sciatic nerve (Gartner et al., 2013). The differentiation into neuroglial-like cells of the MSCs from Wharton's jelly was tested based on the expression of typical neuronal markers such as growth-associated protein-43 (GAP-43), glial fibrillary acidic protein (GFAP), and neuronal nuclei (NeuN). Undifferentiated MSCs were negatively labeled GFAP, GAP-43, and NeuN. After 96 hours of differentiation, the attained cells were positively stained for glial protein GFAP and for the growth-associated protein GAP-43. All nuclei of neuroglial-like cells were also labeled with the neuron-specific nuclear protein called NeuN showing that differentiation of MSCs into neuroglial-like cells was successfully achieved for MSCs obtained from UCT cryopreserved ($N=52$) and for the PromoCell MSC cell line (Gartner et al., 2013).

## 3. *IN VIVO* TESTING IN THE RAT SCIATIC NERVE MODEL

### 3.1. Surgery technique and the importance of a standardized injury

The most frequently used animal model for studying the peripheral nerve regeneration is the rat because of the widespread availability of these animals

as well as the distribution of their nerve trunks, which is similar to humans (Mackinnon et al., 1985). The rat sciatic nerve is still by far the most employed experimental model as it also provides a nerve trunk with adequate length and space at the mid-thigh for surgical manipulation and/or introduction of grafts or tube-guides (van Neerven et al., 2012) (Fig. 4.5). And at same time, this experimental model provides a very realistic testing bench for lesions involving plurifascicular mixed nerves with axons of different size and type competing to reach and reinnervate distal targets (Mackinnon et al., 1985). Peripheral nerve injuries are classified in two major groups, which are the crushing injuries (axonotmesis) and the complete nerve section (neurotmesis) with or without loss of nerve tissue (Almeida-Porada et al., 2005; Chaudhry, Glass, & Griffin, 1992; Stoll, Griffin, Li, & Trapp, 1989). The axonotmesis injury is less severe and is often used for studies concerning the physiology mechanisms of regeneration, while the neurotmesis injury is mostly used in surgical strategy and implementation of biomaterials associated with cellular systems studies (Luís, 2008). The choice of an appropriate animal model is fundamental for preclinical experiments and should be performed before the clinical trials and compassive treatments in humans (Maurício et al., 2011). Nowadays, animal welfare is crucial and not negotiable in terms of *in vivo* experiments. Some alternatives to animal experimentation exist, but what concerns cell therapies and regenerative medicine using stem cells and different biomaterials is always the last step of the research line before the clinical application in humans. Our research group has been using the rat sciatic nerve for *in vivo* experiments of peripheral nerve regeneration (Amado et al., 2010, 2008;

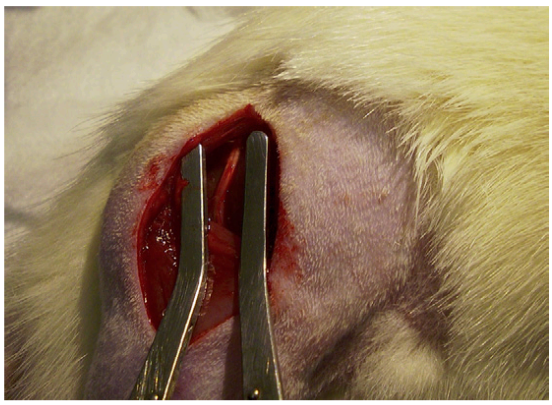

**Figure 4.5** Rat sciatic nerve.

Cheng et al., 2011; Gartner et al., 2012; Gärtner et al., 2012; Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes, et al., 2008; Maurício et al., 2011; Simoes et al., 2010), concerning different therapeutic strategies in axonotmesis (Brohlin et al., 2009) and neurotmesis (Luis et al., 2007) injuries. So, for these *in vivo* testing, Sasco Sprague adult male rats (Charles River Laboratories, Barcelona, Spain) have been used and divided in groups of 6–7 animals each. All procedures had been performed with the approval of the Veterinary Authorities of Portugal, and in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

The animals placed prone under sterile conditions and the skin from the clipped lateral right thigh scrubbed in a routine fashion with antiseptic solution. Under deep anesthesia (ketamine 9 mg/100 g; xylazine 1.25 mg/100 g, atropine 0.025 mg/100 g body weight, intramuscular), the sciatic nerve is exposed unilaterally through a skin incision extending from the greater trochanter to the mid-thigh followed by a muscle-splitting incision. The standard crush injury (axonotmesis lesion) is performed by a nonserrated clamp (Institute of Industrial Electronic and Material Sciences, University of Technology, Vienna, Austria), exerting a constant force of 54 N for a period of 30 s, 10 mm above the bifurcation into tibial and common peroneal nerves inducing a 3-mm axonotmesis lesion, since the published experimental work from Luis and collaborators in 2007 (Luís et al., 2007) (Fig 4.6A). To test the scaffold (biomaterial associated or not to the cellular system) therapeutic effect in the nerve regeneration after crush, the axonotmesis lesion of 3 mm performed was enwrapped. Figure 4.6B shows the crush injury enwrapped with a PVA tube-guide loaded with CNTs. For the neurotmesis lesion, a transaction injury is usually performed using straight microsurgical scissors and the surgical procedure is performed with the aid of an M-650 operating microscope (Leica Microsystems, Wetzlar, Germany). In both models, the nerve injury is performed at a level as low as possible, in general, immediately above the terminal nerve ramification, considering always individual anatomical differences. The reconstruction of the injured nerve after neurotmesis by an end-to-end suture, implies cooptation with 7/0 monofilament nylon sutures of the two injured nerve endings performed under magnification. In the rats in which tube-guides were used for the neurotmesis injured sciatic nerve, the proximal and distal nerve stumps were inserted 3 mm into the tube-guide and held in place, maintaining a nerve gap of 10 mm, with two epineurial sutures using 7/0 monofilament nylon, respectively (Fig. 4.7A–C). For the

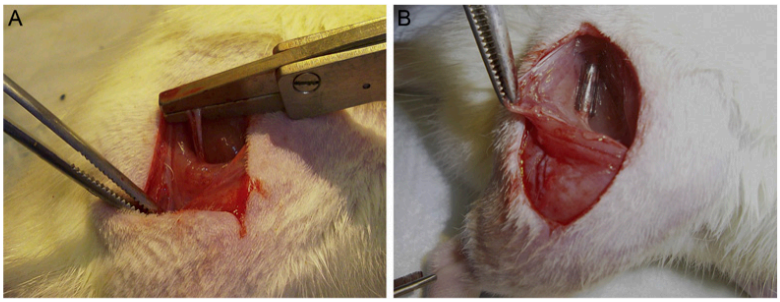


**Figure 4.6** The standard crush injury (axonotmesis lesion) is performed by a nonserrated clamp exerting a constant force of 54 N for a period of 30 s, inducing a 3-mm axonotmesis lesion (panel A). Crush injury enwrapped with a PVA tube-guide loaded with CNTs (panel B).

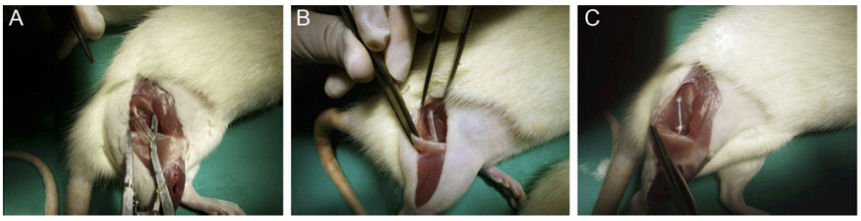


**Figure 4.7** In the rats in which a PVA tube-guide was used for the neurotmesis injured sciatic nerve, the proximal (panel A) and distal nerve stumps (panel B) were inserted 3 mm into the tube-guide and held in place, maintaining a nerve gap of 10 mm, with two epineurial sutures using 7/0 monofilament nylon (panel C).

group of animals in which an autologous graft procedure was tested, the sciatic nerve was transected immediately above the terminal nerve ramification and at a 10 mm distal point. The nerve graft obtained, with a length of 10 mm, was inverted at 180° and sutured with 7/0 monofilament nylon. Normally the muscle and skin are closed with 4/0 resorbable sutures. An antibiotic (enrofloxacin, Alsir® 2.5%, 5 mg/kg b.w., subcutaneously) is always administered to prevent any infections. To prevent autotomy, a deterrent substance must be applied to the rats' right foot (Sanada et al., 2012; Wood et al., 2012). There was no need of administrating immunosuppressive treatment to the experimental animals during the entire healing period after the surgical procedure.

## 3.2. Functional assessment

Experiments on peripheral nerve regeneration are often performed on the rat sciatic nerve model (Dellon & Mackinnon, 1989). Research on

peripheral nerve injury needs to combine both functional and morphological assessment. It is not generally agreed which type of evaluation tool is the most useful descriptor of functional recovery; for this reason, the use of different methods for an overall assessment of nerve function has been recommended by several investigators (Morris, Hudson, & Weddell, 1972). In our lab, we perform a variety of independent evaluation tools in order to understand and estimate the potential therapeutic benefit of a nerve repair strategy.

After injury and treatment of animals, follow-up results are very important for analysis of functional recovery. Animals have been tested preoperatively (week 0), and every week during 12 and 20 weeks, for axonotmesis and neurotmesis of the rat sciatic nerve, respectively.

For SFI, animals are usually tested in a confined walkway that they cross, measuring 42 cm long and 8.2 cm wide, with a dark shelter at the end. Several measurements must be taken from the footprints: (i) distance from the heel to the third toe, the print length (PL); (ii) distance from the first to the fifth toe, the toe spread (TS); and (iii) distance from the second to the fourth toe, the intermediary toe spread (ITS). In the SSI, only the parameters TS and ITS, are measured. For SFI and SSI, all measurements are taken from the experimental (E) and normal (N) sides. Prints for measurements are chosen at the time of walking based on preciseness, clarity, and completeness of footprints. The mean distances of three measurements are used to calculate the following factors (dynamic and static):

$$\text{Toe spread factor (TSF)} = \frac{(\text{ETS} - \text{NTS})}{\text{NTS}} \tag{4.1}$$

$$\text{Intermediate toe spread factor (ITSF)} = \frac{(\text{EITS} - \text{NITS})}{\text{NITS}} \tag{4.2}$$

$$\text{Print length factor (PLF)} = \frac{(\text{EPL} - \text{NPL})}{\text{NPL}} \tag{4.3}$$

SFI is calculated as described by Bain et al. (1989) according to the following equation:

$$\begin{aligned} \text{SFI} &= \frac{-38.3}{\text{NPL}} + \frac{109.5(\text{ETS} - \text{NTS})}{\text{NTS}} + \frac{13.3(\text{EIT} - \text{NIT})}{\text{NIT}} - 8.8 \\ &= (-38.3 \times \text{PLF}) + (109.5 \times \text{TSF}) + (13.3 \times \text{ITSF}) - 8.8 \end{aligned} \tag{4.4}$$

For SFI and SSI, an index score of 0 is considered normal and an index of −100 indicates total impairment. When no footprints are measurable,

the index score of −100 is given. In each walking track, three footprints should be analyzed by a single observer, and the average of the measurements is used in SFI calculations.

Among the large variety of available motor and nociceptive tests, the EPT and the WRL, respectively, have been proven to be reliable, valid and highly efficient methods to determine functional recovery following sciatic nerve injury (Gartner et al., 2013). For EPT test, the affected and normal limbs should be tested at least three times, with an interval of 2 min between consecutive tests, and the three values are averaged to obtain a final result. The normal (unaffected limb) EPT (NEPT) and experimental EPT (EEPT) values are incorporated into an equation (Eq. 4.5) to derive the percentage of functional deficit, as described in the literature (Koka & Hadlock, 2001):

$$\%\ \text{Motor deficit} = \left[\frac{(\text{NEPT} - \text{EEPT})}{\text{NEPT}}\right] \times 100 \qquad (4.5)$$

The nociceptive WRL was adapted from the hotplate test developed by Masters et al. (1993). Normal rats withdraw their paws from the hotplate within 4 s or less. The cutoff time for heat stimulation is set at 12 s to avoid skin damage to the foot.

The restoration of locomotor activity following damage of the nervous system has emerged as one of the most pressing and challenging problem in clinical neuroscience. Many patients with neurological injuries, like peripheral nerve or spinal cord injuries suffer from muscle weakness and loss of independent joint control, often resulting in gait disorders. During the past 10–15 years, exciting work is being carried out on rat gait analysis that may significantly alter the future of peripheral nerve research (Costa, Simoes, Mauricio, & Varejao, 2009). Indeed, the use of biomechanical parameters has given valuable insight into the effects of the sciatic denervation/reinnervation, and thus represents an integration of the neural control acting on the ankle and foot muscles, which is very useful and accurate to evaluate different therapeutic approaches (Varejao, Cabrita, Geuna, et al., 2003; Varejao, Cabrita, Meek, et al., 2003; Varejao, Melo-Pinto, Meek, Filipe, & Bulas-Cruz, 2004). It is important to realize that the number of kinematic variables (positions, velocities, and accelerations) required to describe one-step cycle is very high. Therefore, it is only through high-speed digital cameras that we can achieve a full kinematic description during gait (Costa et al., 2009).

Ankle kinematics analysis has been carried out prior nerve injury, at week 2 and every 4 weeks during the 12 or the 20-week follow-up time,

for axonotmesis and neurotmesis lesions, respectively. The motion capture is performed with two digital high speed cameras (Oqus, Qualysis®) at a rate of 200 images per second, and Qualysis Track Manager software (QTM, Qualysis®). The cameras operate on an infra-red light frequency ensuring a high level of accuracy on the determination of reflective marker position and a position residual of less than 2.7 mm was obtained. Cameras are usually positioned to not recorder significant signal deflection during the test and three reflective markers are placed at the skin of the rat right hindlimb at the proximal edge of the tibia, the lateral malleolus and the fifth metatarsal head. Advanced analysis of the 2D movement (sagittal plan) data is usually performed with Visual3D software (C-Motion®, Inc.). The rats' ankle angle is determined using the scalar product between a vector representing the foot and a vector representing the lower leg. With this model, positive and negative values of position of the ankle joint ($\Theta°$) indicate dorsiflexion and plantarflexion, respectively. For each step cycle, the following time points are identified: midswing, midstance, initial contact (IC) and toe-off (TO) (Gartner et al., 2013) and are time normalized for 100% of step cycle. The normalized temporal parameters are averaged over all recorded trials. Angular velocity of the ankle joint ($\Omega$ °/s) is also determined where negative values correspond to dorsiflexion. A total of at least six walking trials for each animal with stance phases lasting between 150 and 400 ms are considered for analysis, since this corresponds to the normal walking velocity of the rat (20–60 cm/s) (Gartner et al., 2013). The motion capture is performed when the animals walk on a Perspex track with length, width, and height of respectively 120, 12, and 15 cm. In order to ensure locomotion in a straight direction, the width of the apparatus is adjusted to the size of the rats during the experiments and the rats are daily trained for 2 weeks before the surgery, in order to walk on the Perspex corridor.

Individual joint kinematics either in control or nerve-injured animals is characterized by high variability, with notable differences between different animals and even from step to step (Jacobson & Guth, 1965). Such high level of variability, which seems to be an intrinsic property of normal quadruped walking, seriously affects the precision of joint kinematic measures of functional recovery after nerve injury. Reducing this variability is a challenge for efficient use of walking analysis to assess functional recovery. Attempts to overcome this limitation include constraining walking velocity by using treadmill walking instead of self-paced locomotion (Gartner et al., 2013). This, of course, is likely to reduce step-by-step variability in joint kinematics but has the disadvantage of requiring expensive equipment and limits the

possibility of combining kinematic analysis with other data, such as ground reaction forces. Other possibilities look at a global, limb-level movement analysis as an alternative to individual joints kinematics (Jacobson & Guth, 1965; Pereira et al., 2013). To better assess hindlimb joint kinematics during walking, we recently analyzed hip, knee, and ankle joint kinematics during recovery of less severe sciatic nerve crush injury, using a more sophisticated motion capture system to track the motion of reflective markers attached to the rat hindlimb (unpublished data). Recently a segmental kinematic analysis using both planar angles computation and a tridimensional (3D) reconstruction of the rat hindlimb was also performed, regarding the morphology and the movement of each segment. Seven rats without any peripheral injury were evaluated for natural overground walking, and motion capture of the right hindlimb was collected with an optoelectronic system while the animals walked in the track. 3D biomechanical analyses were carried out and hip, knee, ankle, and metatarsophalangeal joint angular displacements were calculated. A comparison between planar and 3D segmental kinematic analysis using a tridimensional reconstruction of the rat hindlimb demonstrated that different joints have different motion patterns within motion planes, probably related with physiological constraints and muscle actions. A major indication of the need for an anatomical reference frame kinematic analysis is supported by the knowledge that neuromuscular diseases are related to important clinical signs or motor deficits that should be observed, qualified, and quantified (Harley et al., 2006). On the other hand, systematic changes in the biomechanical and movement control constraints of the locomotor task, such as using up- and down-slope walking might also increase the accuracy of walking analysis within the context of peripheral nerve research (Pereira et al., 2013). Walking analysis is a promising method to assess functional recovery after hindlimb nerve injury. However, in order to provide accurate measures of functional recovery, walking analysis after hindlimb peripheral nerve injury will have to evolve from simply analyzing ankle kinematics to reach a full biomechanical description of hindlimb motion including analysis of hip, knee, and ankle joints. Further refinements of walking analysis in the field of peripheral nerve research using the rat model will probably include the combined use of joint kinematics, ground reaction forces, and electromyographical data of muscle activity (Harley et al., 2006; Maurício et al., 2011).

### 3.3. Morphologic assessment

It has been recently pointed out that morphological analysis is the far most common method for the study of peripheral nerve regeneration (Raimondo

et al., 2009). Actually, the investigation of nerve morphology can give us important information on various aspects of the regeneration processes which relates with nerve function (Geuna et al., 2009). Although different types of fixatives can be used for peripheral nerve histology, the nerve samples are fixed in a solution of 2.5% purified glutaraldehyde (Histo-line Laboratories S.R.L., Milano, Italy) and 0.5% saccarose (Merck, Darmstadt, Germany) in 0.1 M Sörensen phosphate buffer, pH 7.4, for 6–8 h. Nerves are then washed and stored in 0.1 M Sörensen phosphate buffer added with 1.5% saccarose at 4–6 °C prior to embedding. Sörensen phosphate buffer is made with 56 g di-potassium hydrogen phosphate 3-hydrate ($K_2HPO_4$-$3H_2O$) (Fluka, Buchs, Switzerland) and 10.6 g sodium di-hydrogen phosphate 1-hydrate ($NaH_2PO_4$-$H_2O$) (Merck, Darmstadt, Germany) in 1 liter of doubly-distilled water. Just before the embedding, nerves are washed for few minutes in the storage solution and then immersed for 2 h in 2% osmium tetroxide (Sigma, St. Louis, MO) in the same buffer solution. The specimens are then carefully dehydrated in passages in ethanol and embedded in Glauerts' mixture of resins, which is made of equal parts of Araldite M and the Araldite Härter, HY 964 (Merck, Darmstad, Germany). At the resin mixture, 2% of accelerator 964, DY 064 is added (Merck, Darmstad, Germany). Finally, the plasticizer (0.5% of dibutylphthalate) is added to the resin. In Stefano Geuna laboratory (Department of Clinical and Biological Sciences, University of Turin, Italy), histomorphometry (stereology) is carried out on toludine-blue-stained semi-thin sections (2.5 micron-thick) of nerve samples using a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany). We adopt a final magnification of 6600× in order to enable accurate identification of myelinated nerve fibers. A 2D-disector method, (Raimondo et al., 2009) is finally used for estimating the total number of myelinated fibers ($N$), the mean diameter of fiber ($D$) and axon ($d$) as well as mean $[(D-d)/2]$ and $g$-ratio ($D/d$).

## 4. DISCUSSION AND FINAL REMARKS

Despite great progress in the fields of tissue engineering and stem cell therapy, translational and preclinical studies are required to accelerate the clinical application of scaffolds as an alternative to autologous nerve grafts for peripheral nerve repair. The PNS is able to regenerate after traumatic injury, but most frequently, the functional outcomes following damage are limited and poor. Nowadays, most tissue-engineered nerve grafts are

composed of a neural scaffold prepared with a variety of synthetic or natural biomaterials through well-defined fabrication techniques. Introduction of support cells, an important biochemical cue, represents an optimal way for constructing tissue-engineered nerve grafts with enhanced ability to repair extended peripheral nerve defects. MSCs are multipotent cells that have been used in studies of peripheral nerve regeneration and have yielded promising results. The aim of our research group for the past 10 years has been exploring the therapeutic value of human UC Wharton's jelly-derived MSCs both *in vitro* and *in vivo*, associated to several tube-guides of natural or absorbable synthetic biomaterials on a rat sciatic nerve axonotmesis experimental model. Undifferentiated MSCs from human UC Wharton's jelly have been expanded and have exhibited a normal star-like shape with a flat morphology in culture. To prevent the possibility of eventual mutations due to expansion artifacts, Giemsa-stained metaphases of these cells were analyzed for numerical aberrations. The karyotype was determined in a completely analyzed G-banding metaphase and no structural alterations were found. The karyotype analysis to the MSCs cell line derived from human Wharton jelly demonstrated that this cell line has not neoplasic characteristics and was stable during the cell culture procedures in terms of number and structure of the somatic and sexual chromosomes (Gartner et al., 2012). The MSCs from Wharton's Jelly were differentiated into neuroglial-like cells in the presence of neurogenic culture medium during 96 h. The MSCs became exceedingly long and there was a formation of typical neuroglial-like cells with multibranches and secondary branches. The differentiation was also tested based on the expression of typical neuronal markers such as GFAP, GAP-43, and NeuN by neural-like cells attained from HwMSCs. Undifferentiated MSCs were negatively labeled to GFAP, GAP-43, and NeuN and after 96 h of differentiation the attained cells were positively stained for glial protein GFAP and for the growth-associated protein GAP-43. All nucleus of neuroglial-like cells were also labeled with the neuron-specific nuclear protein called NeuN showing that differentiation was successfully achieved (Gartner et al., 2012). The *in vitro* expansion and differentiation of MSCs for clinical cell-based therapy is a very expensive and long process that needs standardization. Although preclinical and clinical data demonstrated the safety and effectiveness of MSCs therapy in some pathologies such as neurological, there are still questions surrounding the mechanism of action. MSCs' maintenance and differentiation to neuroglial-like cells depends on metabolic modulation. *In vitro*, glucose is the most widely used substrate for the generation of ATP, which is

essential for cell growth and maintenance. It has been proposed that cells undergoing high proliferation rates depend on glycolysis to generate ATP, known as the Warburg effect (Gartner et al., 2012, 2013). Our results showed that during expansion, the undifferentiated MSCs consume glucose and produce high concentration of lactate as a metabolic sub product, which is consistent with the Warburg effect and glycolysis stimulation. MSCs do not require oxidative phosphorylation to survive as alternative, hypoxia extends the lifespan, increases their proliferative ability, and reduces differentiation (Gartner et al., 2012, 2013). We recently *in vivo* tested using the rat model, the efficacy of several natural and synthetic biomaterials associated with cellular systems including the MSCs isolated from the UC Wharton's jelly in the treatment of sciatic nerve axonotmesis and neurotmesis injuries (Amado et al., 2008; Gartner et al., 2012; Maurício et al., 2011). Following transection, axons show staggered regeneration and may take substantial time to cross the injured site and enter the distal nerve stump (Gartner et al., 2012, 2013). However, delayed axonal elongation might be caused by growth inhibition originated from the distal nerve itself, growth-stimulating influences may overcome axons stagger. As a potential source of growth-promoting signals, MSCs transplantation is expected to give a positive outcome. Our results showed that the use of either undifferentiated or differentiated MSCs in axonotmesis and neurotmesis lesions boosted the recovery of sensory and motor function. In both cell-enriched experimental groups, we observed that the myelin sheath was thicker; this suggests that MSCs might apply their positive effects on SCs, the key element in Wallerian degeneration and the following axonal regeneration (Gartner et al., 2012, 2013). Also results from *in vivo* testing previously performed showed that infiltration of MSCs from the Wharton's jelly or the combination of chitosan type III membrane enwrapment and MSCs enrichment after nerve crush injury provide an advantage to post-traumatic nerve regeneration (Amado et al., 2008; Costa et al., 2009; Gartner et al., 2012; Maurício et al., 2011). Chitosan type III was developed as a hybrid of chitosan by adding GPTMS. A synergistic effect of an extra permeability and physicochemical properties of chitosan type III and the presence of silica ions may be responsible for the good results in post-traumatic nerve regeneration promotion observed in the sciatic nerve after axonotmesis and neurotmesis suggesting that this biomaterial may not just work as a simple mechanical device but instead may induce nerve regeneration (Amado et al., 2008; Simoes et al., 2010). The neuroregenerative properties of chitosan type III may be explained by the effect on SCs proliferation, axon elongation,

and myelinization (Amado et al., 2008; Gärtner et al., 2012; Simoes et al., 2010). Our data also showed that PLC does not deleteriously interfere with the nerve regeneration process, as a matter of fact, the information on the effectiveness of PLC membranes and tube-guides for allowing nerve regeneration was already provided experimentally and with patients (Maurício et al., 2011). The MSCs from the Wharton's jelly may be a valuable source in the repair of the PNS with capacity to differentiate into neuroglial-like cells. The transplanted MSCs are also able to promote local blood vessel formation and release the neurotrophic factors brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) (Gartner et al., 2012; Gärtner et al., 2012). Previous results obtained by our research group using N1E-115 cells *in vitro* differentiated into neuroglial-like cells to promote regeneration of axonotmesis and neurotmesis lesions in the rat model showed that there was no significant effect in promoting axon regeneration and, when N1E-115 cells were cultured inside a PLGA scaffold used to bridge a nerve defect, they can even exert negative effects on nerve fiber regeneration. The presence of transplanted N1E-115 cells in nerve scaffolds competing for the local blood supply of nutrients and oxygen and by space-occupying effect could have hindered the positive effect of local neurotrophic factor release leading a negative outcome on nerve regeneration. Thus, N1E-115 cells did not prove to be a suitable candidate cellular system for treatment of nerve injury after axonotmesis and neurotmesis and their application is limited only to research purposes as a basic scientific step for the development of other cell delivery systems, due to its neoplasic origin (Amado et al., 2010, 2008; Luis, Rodrigues, Geuna, Amado, Shirosaki, et al., 2008; Luis, Rodrigues, Geuna, Amado, Simoes, et al., 2008; Simoes et al., 2010). The MSCs isolated from the Wharton's jelly and delivered through PLC and chitosan type III membranes might be a potentially valuable tool to improve clinical outcome especially after trauma to sensory nerves, such as digital nerves. Results demonstrated that the use of either undifferentiated or neuroglial-like differentiated MSCs enhanced the recovery of sensory and motor function of the rat sciatic nerve in axonotmesis and neurotmesis injuries (Gartner et al., 2012; Gärtner et al., 2012). The myelin sheath was thicker in the regenerated nerves, suggesting that MSCs might exert their positive effects on SCs, the key element in Wallerian degeneration and the following axonal regeneration (Gartner et al., 2013). Many researchers believe that the implanted MSCs exert neurotrophic functions by producing an array of soluble factors or via a direct cell-to-cell contact rather than replacement of damaged nerve

tissues and cells. We have been using undifferentiated MSCs with positive results concerning the functional and morphologic recovery of the nerve after axonotmesis and neurotmesis injuries, in an attempt to diminish the possible adverse effects related to an *in vitro* induced differentiation of the MSCs. Moreover, direct use of MSCs without additional treatments is more favorable to clinical applications owing to simplify the treatments, which implies a less financial burden. In addition, the MSCs isolate from the Wharton's jelly represent a noncontroversial source of primitive mesenchymal progenitor cells that can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses, including nerve injuries like axonotmesis and neurotmesis. The time and temperature of the transport (and the saline solution used for that transport) of the UC units from the hospital/clinic to the laboratory is crucial for a successful outcome considering MSCs isolation and proliferation from fresh and cryopreserved tissue. It is highly recommended that the transport from the clinic to the hospital should be refrigerated, and the UC units should be immediately immersed in a sterile saline solution like HBSS or DPBS.

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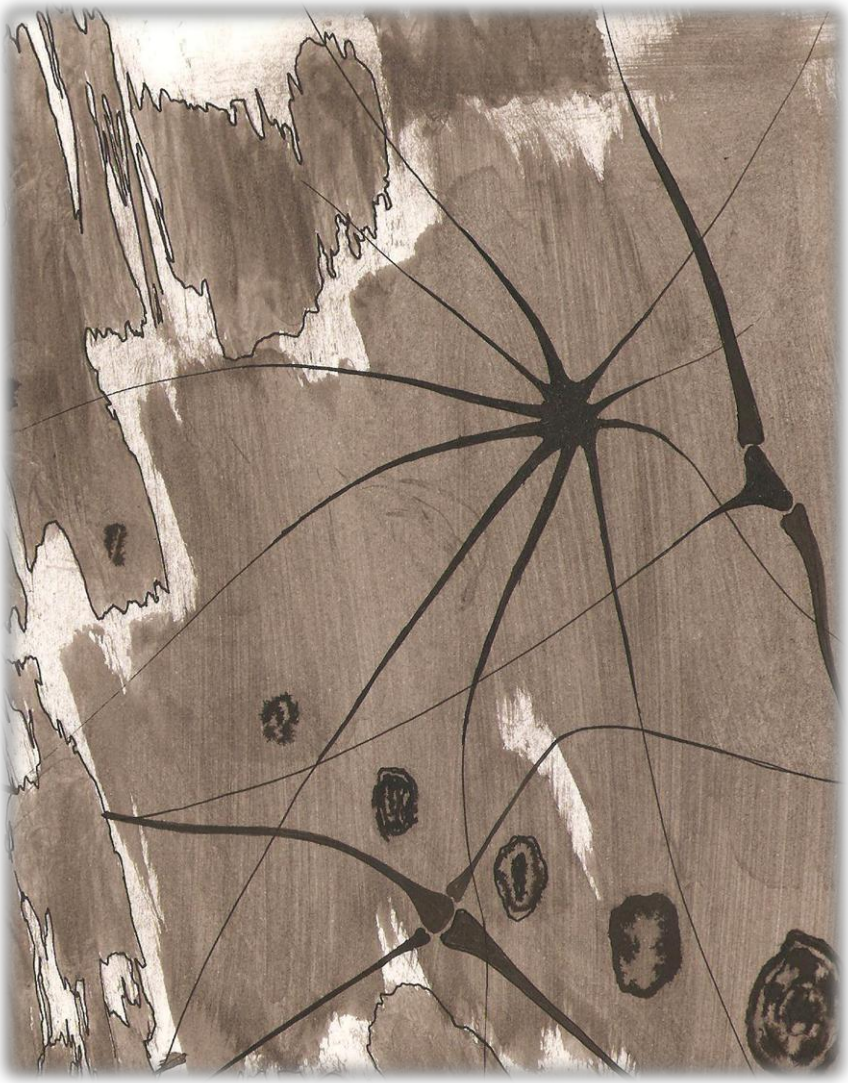

## Capítulo III - Trabalho experimental

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### 1. Objetivos do estudo e organização dos trabalhos experimentais

O estudo realizado no âmbito deste Doutoramento teve por objetivos principais:

- Desenvolvimento e caracterização (físico-química e mecânica) *in vitro* de um biomaterial (PVA) e sua associação com polímeros condutores e outros iões metálicos, para obtenção de membranas e tubos-guia com alta condutividade elétrica para reconstrução cirúrgica de lesões de axonotmese e neurotmese;
- Estudo *in vivo* da aplicação de tubos-guia e membranas de PVA melhorados com polímeros condutores, em lesões de axonotmese e neurotmese, recorrendo ao modelo animal do nervo ciático do rato;
- Estudo *in vivo* da incorporação de sistemas celulares (MSC's) em tubos-guia de PVA e seus resultados em lesões de neurotmese recorrendo ao modelo animal do nervo ciático do rato;
- Aplicação clínica do PVA em casos clínicos de animais de companhia em medicina veterinária.

Tendo em conta os objetivos traçados, a primeira fase foi o desenvolvimento de tubos-guia do biomaterial (PVA), tornando-o passível de ser aplicado em lesões de nervo periférico, melhorando as suas qualidades físicas e mecânicas para aplicação em nervo periférico.

A segunda fase foi a associação do PVA com moléculas que aumentam a condutividade elétrica, caracterização dos diferentes tubos-guia obtidos e selecionar as moléculas a associar ao PVA para os estudos *in vivo* posteriores.

A terceira fase foi a aplicação dos tubos escolhidos em lesões de axonotmese e avaliação dos resultados, para posterior escolha do tubo-guia a associar ao sistema celular (MSCs isoladas da geleia de Wharton do cordão umbilical).

A quarta fase foi a aplicação dos diferentes tubos-guia em lesões de neurotmese, onde num dos grupos experimentais se utilizou o biomaterial selecionado associado a MSCs.

Ao longo de todo o Doutoramento, dado a continuação em paralelo da atividade clínica na área da cirurgia de animais de companhia, foram angariados casos clínicos em que a aplicação da medicina regenerativa poderia apresentar-se como uma melhor solução para a sua resolução.

## 2. Resultados

Os resultados obtidos no âmbito destes trabalhos de Doutoramento foram publicados em revistas internacionais com arbitragem científica.

### 2.1. Produção e caraterização do biomaterial à base de PVA - *Preparation and characterization of electrical conductive PVA based materials for peripheral nerve tube-guides*

O desenvolvimento do método de produção de tubos-guia, a sua caracterização físico-química e mecânica e a avaliação da sua citocompatibilidade encontram-se descritos no artigo científico publicado:

Gonçalves, C., **Ribeiro, J.**, Pereira, T., Luís, A. L., Maurício, A. C., Santos, J. D., & Lopes, M. A. (2016). **Preparation and characterization of electrical conductive PVA based materials for peripheral nerve tube-guides**. *Journal of Biomedical Materials Research Part A*: 2016: 104A:1981-1987

# Preparation and characterization of electrical conductive PVA based materials for peripheral nerve tube-guides

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# Preparation and characterization of electrical conductive PVA based materials for peripheral nerve tube-guides

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**Abstract:** Peripheral nerve regeneration is a serious clinical problem. Presently, there are several nerve tube-guides available in the market, however with some limitations. The goal of this work was the development of a biomaterial with high electrical conductivity to produce tube-guides for nerve regeneration after neurotmesis injuries whenrver an end-to-end suture without tension is not possible. A matrix of poly(vinyl alcohol) (PVA) was used loaded with the following electrical conductive materials: COOH-functionalized multiwall carbon nanotubes (MWCNTs), poly(pyrrole) (PPy), magnesium chloride ($MgCl_2$), and silver nitrate ($AgNO_3$). The tube-guide production was carried out by a freezing/thawing process (physical crosslinking) with a final annealing treatment. After producing the tube-guide for nerve regeneration, the physicochemical characterization was performed. The most interesting results were achieved by loading PVA with 0.05% of PPy or COOH- functionalized CNTs. These tubes combined the electrical conductivity of carbon nanotubes (CNTs) and PPy with the biocompatibility of PVA matrix, with potential clinical application for nerve regeneration. © 2016 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00B:000–000, 2016.

**Key Words:** poly(vinyl alcohol), carbon nanotubes, poly(pyrrole), nerve guide, tissue engineering

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## INTRODUCTION

Peripheral nerve regeneration is a serious clinical problem in many patients, with unsatisfactory functional recovery.[1,2] Peripheral nerve injury is mostly due to trauma, with up to 300,000 cases each year in Europe alone and in the United States. As a matter of fact, ~200,000 patients each year are referred to neurosurgery due to neurotmesis injuries.[3] Many strategies have been applied for the repair of peripheral nerve lesions, with the common goals of directing the regenerating nerve fibers into the proper distal endoneural tubes and improving the prospects of axonal regeneration and functional recovery.[4] The treatment and recovery of peripheral nerve injuries is still one of the most challenging tasks in neurosurgery.[5] Nowadays, when a nerve is damage and there is a neurotmesis injury with a gap, it can be repaired using a nerve tube-guide whenever it is not possible to perform an end-to-end suture without tension or if there isn't a graft available. Natural and synthetic biomaterials have been used in tube-guides, being the later subdivided into two major groups: biodegradable and nonbiodegradable.[6] The development of tube-guides is a consequence of the limitations inherent in the use of grafts, in terms of length, diameter and type of fiber, preventing damage to the donor local.[7] The desired physical properties of a nerve conduit include; a biodegradable and porous channel wall; the ability to deliver bioactive factors; the incorporation of support cells; an internal oriented matrix to support cell migration; intraluminal channels to mimic the structure of nerve fascicles; and electrical activity.[8]

In this work the electrical conductivity of different materials based on poly(vinyl alcohol) (PVA) matrix loaded with non-functionalized multiwall carbon nanotubes (MWCNTs), poly(pyrrole) (PPy), silver nitrate ($AgNO_3$) and magnesium

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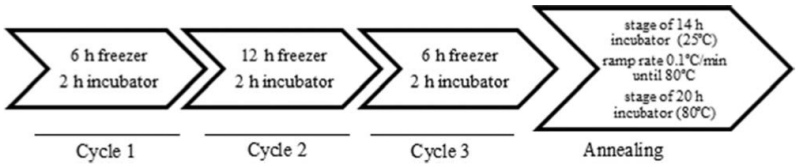


**FIGURE 1**. The freezing/thawing process and the annealing treatment used for tube guide preparation.

chloride ($MgCl_2$) were analyzed, being the ones with the best results submitted to a more detailed characterization of their properties aiming their use as a nerve tube-guide.

## MATERIAL AND METHODS

### Production of nerve guide tubes

Synthetic biodegradable tubes of PVA (Aldrich, Mowiol 10–98) and PVA loaded with COOH- functionalized multiwall carbon nanotubes (Nanothinx, NTX5, MWCNTs 97% -COOH) and non-functionalized multiwall carbon nanotubes (NTX1, MWCNTs 97%), poly(pyrrole) (PPy) (Aldrich, 10–40 S/cm of conductivity), $MgCl_2$ (Merck, TA361902 802) and $AgNO_3$ (Sigma-Aldrich, ≥99% purity), were prepared using a casting technique to a silicone mold.

A 15% (%w/v) aqueous solution of PVA was prepared. Then the solution of PVA was mixed with 0.05% and 0.1% (%w/v) of COOH- functionalized and non-functionalized CNTs, 0.05% and 0.1% of PPy, 0.05 and 1% of $AgNO_3$, and 5% and 10% of $MgCl_2$.

The tube-guides were produced by freezing/thawing process. The treatment consisted in three cycles of freezer (−30°C)/incubator (25°C), and a final annealing treatment, that started with a stage of 14 h on an incubator (25°C) followed by a ramp rate of 0.1°C/min until 80°C, and finally, a stage of 20 h at 80°C as described in Figure 1. The tube-guides were hydrated during 2 h and stayed in sterile distilled water until use.

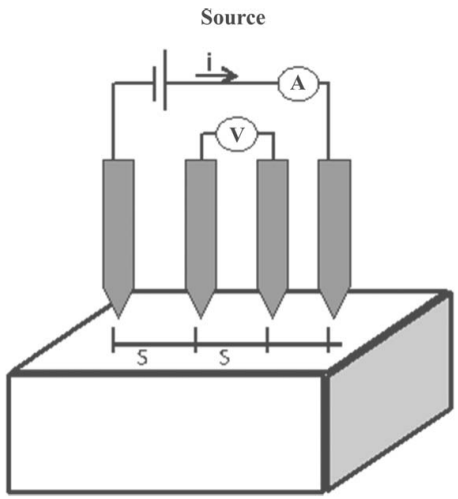


**FIGURE 2**. Arrangement for resistivity measurements by four point method. The letters "s" represents the distance between points that are known; A, ammeter; V, voltmeter; I, current intensity - (adapted from Girotto and Santos 2002).

### Physicochemical and mechanical characterization

***Electrical conductivity analysis.*** The electrical conductivity was measured using a four point method. Four electrodes were placed linearly, the current was injected through two electrodes and it was measured the tension on the other two. The probes to monitor the current and voltage were placed on a special stand with the tips in line at an equal distance from each other.[9] Figure 2 illustrates the experimental arrangement for the four probes tips.

The resistivity was obtained measuring a small sample (15 mm x 4 mm) of each fresh material (simple PVA, PVA loaded with COOH- functionalized and nonfunctionalized CNTs, PVA loaded with PPy, PVA loaded with $AgNO_3$ and PVA loaded with $MgCl_2$) and dried immediately previously to the measurements (eight samples of each material). When the tips are equally spaced (s is equal) the resistivity (Ω m) is measured using the Eq. (1), where $\rho$ is the resistivity, $V$ is the voltage measured, and $I$ the current injected. The Eq. (2) represents the conductivity ($\sigma$, in S/m) that is the inverse of resistivity[10]:

$$\rho = 2\pi s \times V/I \quad (1)$$

$$\sigma = 1/\rho \quad (2)$$

***Differential scanning calorimetry (DSC) analysis.*** The melting temperature ($T_m$), glass transition temperature ($T_g$) and enthalpy was obtained for simple PVA, PVA loaded with COOH- functionalized CNTs, and PVA loaded with PPy using about 10 mg of sample for each test. The samples were heated from 25 to 300°C at 5°C/min, kept at 300°C for 5 min and cooled until 25°C at 20°C/min under argon atmosphere. Enthalpy of fusion ($\Delta H$, J/g) value was calculated by numerical integration of the area under the melting peak. This was divided by 155 J/g, which is the theoretical enthalpy for ~100% crystalline PVA, to give the crystallinity level, $X_{Cr}$.[11]

$$X_{Cr} = \frac{\Delta H}{\Delta H_c} \times 100\% \quad (3)$$

The measurements were performed using a SETARAM Instrumentation equipment, LABSYS™ TG.

***Fourier transform infrared spectroscopy (FTIR) analysis.*** The identification of functional groups of simple PVA, PVA loaded with COOH- functionalized CNTs and PVA loaded

**TABLE I. Electrical Conductivity of the Different Nerve Guide Tubes**

| Samples | Conductivity (S/m)*$10^6$ |
|---|---|
| PVA 15% (%w/v) | 1.5 ± 0.5 |
| PVA 15% loaded with $AgNO_3$ 0.5% (%w/v) | 23.3 ± 0.3 |
| PVA 15% loaded with $AgNO_3$1% (%w/v) | 34.5 ± 0.4 |
| PVA 15% loaded with $MgCl_2$ 5% (%w/v) | 130.0 ± 0.3 |
| PVA 15% loaded with $MgCl_2$ 10% (%w/v) | 211.0 ± 0.9 |
| PVA 15% loaded with CNTs 0.05% (%w/v) | 689.0 ± 0.5 |
| PVA 15% loaded with CNTs 0.1% (%w/v) | 825.0 ± 1.0 |
| PVA 15% loaded with COOH- CNTs 0.05% (%w/v) | 579.0 ± 0.6 |
| PVA 15% loaded with COOH- CNTs 0.1% (%w/v) | 1250.0 ± 0.7 |
| PVA 15% loaded with PPy 0.05% (%w/v) | 1837.5 ± 0.7 |
| PVA 15% loaded with PPy 0.1% (%w/v) | 2003.4 ± 0.3 |

with PPy was obtained by Fourier Transform Infrared (FTIR) spectroscopy (SHIMADZ IRAffinity-1; diffuse reflectance accessory EasiDiff TM from PIKE Technologies Inc.). The scanning range was 4000–400 $cm^{-1}$, the resolution was 8 $cm^{-1}$, 50 scans.

***X-ray diffraction (XRD) analysis.*** To evaluate the presence of crystal phases, tubes of simple PVA, PVA loaded with COOH- functionalized CNTs and PVA loaded with PPy were analyzed by XRD (Bruker D8 Discover). The data was recorded using a step size of 0.02° and a 0.4 s dwell time.

***Scanning electron microscope (SEM) analysis.*** The morphological characterization of the simple PVA, PVA loaded with COOH- functionalized CNTs, and PVA loaded with PPy was obtained by Scanning Electron Microscope (JEOL JSM 35C Noran Voyager 15 kV). Two different modes were used to observe the sample: secondary electrons with low energy and backscattered electrons. The samples were covered with gold and palladium film under vacuum atmosphere.

***Wettability analysis.*** Water contact angles were measured on different samples: simple PVA, PVA loaded with COOH- functionalized CNTs, and on PVA loaded with PPy. The test was performed using sessile drop method and applying the axisymmetric drop Shape Analysis-Profile (ADSA-P). At least 10 drops of 4 μL of volume were used in order to obtain the average contact angle. This analysis was performing using ultra-pure water (Merck Germany – 1.01262.1000).

***Zeta potential analysis.*** Zeta potential (ζ) of the nerve guide tubes was measured by the steaming potential technique, using a cylindrical cell (Anton Par, Visolab for Surpass version 2.10). This cell is appropriated to measure zeta potential in materials with a diameter higher than 200 μm. The materials were sliced with a length of 20 mm and width of 3 mm.

Zeta potential, a measure of the electrical charge of a surface, is estimated from the voltage difference between the solid surface and the bulk liquid phase. The nerve guide tubes were exposed to an electrolyte KCl 1 m*M*, which contributes to the equilibrium near the surface. The measurements were performed in triplicate for each material, at physiological pH.

***Dynamic mechanical analysis (DMA).*** The assessment of viscoelastic properties of the nerve guide tubes was performed by Dynamic Mechanical Analysis. The test was performed using compression geometry, at 37°C, frequency of 1 Hz and displacement of 0.05 mm. Triplicates measurements were performed (PerkinElmer, DMA 8000 instrument).

### Evaluation of the cytocompatibility using MSCs from the Whartońs jelly

Human MSCs isolated from the Wharton jelly (WJ) of UCT from PromoCell GmbH (C-12971, lot-number: 8082606.7) were cultured and expanded in a humidified atmosphere with 5% $CO_2$ at 37 by replacing the mesenchymal stem cell medium, PromoCell (C-28010) every 48 h. At 80% confluence, normally obtained after 4 days in culture, MSCs were harvested with 0.25% trypsin with EDTA (GIBCO) for further expansion at an initial concentration of $1 \times 10^4$ cells/$cm^2$. Intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) by using dual wavelength spectrofluorometry as previously described[28,29] was measured in Fura-2-loaded MSCs cells. $[Ca^{2+}]_i$ from MSCs cultured without the presence of any biomaterial, subcultured over PVA discs (PVA 15% (%w/v)), over PVA loaded with CNTs (PVA 15% loaded with COOH- CNTs 0.05%) and PVA loaded with PPy (PVA 15% loaded with PPy 0.05%) discs of 10 mm diameter are results obtained from epifluorescence technique confirming that the MSCs did not begin the apoptosis process. The biomaterials tested for cytocompatibility where chosen since these three types of PVA tube-guides (PVA, PVA 15% loaded with COOH- CNTs 0.05%, and PVA 15% loaded with PPy 0.05%) would be further tested *in vivo*.

### Statistical analysis

The results obtained from the physicochemical analysis, were statistically analyzed. The one way ANOVA test was applied. Each result is presented the mean value of measurements and reported with standard deviation (mean values ± SD). Statistical significance was accepted at $p < 0.05$.

## RESULTS

### Physicochemical and mechanical characterization

***Electrical conductivity analysis.*** The electrical conductivity achieved for the tube-guides is listed in Table I. The aim of this analysis was to choose the best electrical conductive load material and its better loading level. The value of 1.5 x $10^{-6}$ S/m for simple PVA tubes was obtained, which is according to bibliography.[12] Tube-guides with different load levels of COOH- functionalized MWCNTs [0.05% and 0.1% (%w/v)] showed similar values, 5.79 x $10^{-4}$ and 12.50 x $10^{-4}$ S/m, respectively. PVA tubes loaded with 0.05% and 0.1% (%w/v) of nonfunctionalized CNTs also showed similar values, 6.89 x $10^{-4}$ and 8.25 x $10^{-4}$ S/m respectively.[13] Regarding to PVA loaded with PPy, the values were similar for the different loads used (1.8375 x $10^{-3}$ and 2.003 x $10^{-3}$ S/m) and are according to reported values.[14] It is important to note that PPy by itself has some conductivity.

**TABLE II. Thermal Parameters Calculated by DSC**

| Sample | $\Delta H$ (J/g) | $T_m$ (°C) | $T_g$ (°C) | Crystallinity (%) |
|---|---|---|---|---|
| PVA 15% (%w/v) | 11.6 ± 0.7 | 215.5 ± 0.3 | 51.2 ± 2.0 | 7.5 |
| PVA 15% loaded with PPy 0.05% (%w/v) | 11.4 ± 0.01 | 213.3 ± 1.0 | 49.7 ± 3.2 | 7.3 |
| PVA 15% loaded with COOH- CNTs 0.05% (%w/v) | 11.5 ± 0.4 | 215.0 ± 0.2 | 48.9 ± 2.1 | 7.4 |

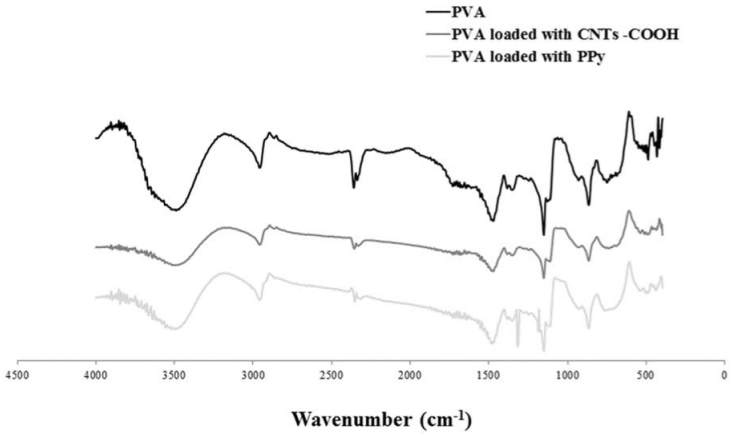


**FIGURE 3.** FTIR spectra of a simple PVA, PVA loaded with COOH-functionalized CNTs and PVA loaded with PPy.

Regarding to the PVA loaded with $MgCl_2$ the values obtained were according to the Mowiol Manual.[15] The PVA loaded with $AgNO_3$ showed a value of 3.45 x $10^{-5}$ and 13.0 x $10^{-5}$ S/m, for additions of 0.5% and 1% (%w/v), respectively.

The best results were obtained for the PVA loaded with PPy (0.05 and 0.1% w/v) and 0.1% (%w/v) of COOH- functionalized and nonfunctionalized CNTs. However, the results obtained for PVA loaded with 0.05% of PPy (%w/v) were very similar to the ones obtained with PVA tube-guides loaded with 0.1% (%w/v) of PPy, and then a load of 0.05% (%w/v) was chosen as the best to prepare electrical conductive tube-guides.

Therefore three materials' compositions to construct tube-guides were chosen for further characterization: PVA, PVA loaded with 0.05% (%w/v) of COOH- functionalized CNTs and PVA loaded with 0.05% (%w/v) of PPy.

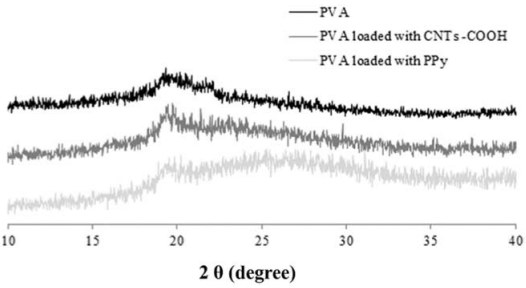


**FIGURE 4.** XRD of a simple PVA, PVA loaded with COOH-functionalized CNTs, and PVA loaded with PPy.

***Differential scanning calorimetry (DSC) analysis.*** The thermal characteristics of PVA and loaded PVA materials were examined by DSC. It is well established that crystalline and amorphous phases in variable amount co-exist in most of the polymeric materials. PVA is one of the partially crystalline polymers exhibiting both the glass transition temperature $T_g$ and a melting point, $T_m$.[16]

The results are presented in Table II. The pure PVA provided a melting temperature around 215.5°C, which is according to bibliography.[17] The other materials showed a melting temperature near the simple PVA. The $T_g$ of dry PVA films has been reported at 85°C; however, in the presence of water and other solvents, this temperature decreases significantly.[18] In fact, the $T_g$ is about 50°C, near of the value found by Moslem et al.[19] The loaded PVA materials showed a $T_g$ near the value of simple PVA probably because of the low loads of PPy and CNTs used. Enthalpy of fusion ($\Delta H$) was calculated, and the percentage of crystallinity was near 7.4% for all analyzed materials.

***Fourier transform infrared spectroscopy (FTIR) analysis.*** Figure 3 shows the FTIR spectra of PVA and PVA loaded with electrical conductive agents. All major peaks related to hydroxyl and acetate groups were observed. The large bands observed between 3550 and 3200 $cm^{-1}$ are linked to the stretching O—H from the intermolecular and intramolecular hydrogen bonds. The vibrational band observed between 2840 and 3000 $cm^{-1}$ refers to the stretching of C—H from alkyl groups and the peaks between 1750 and 1735 $cm^{-1}$ are due to the stretching C=O and C—O from acetate group remaining from PVA.[20] The bands

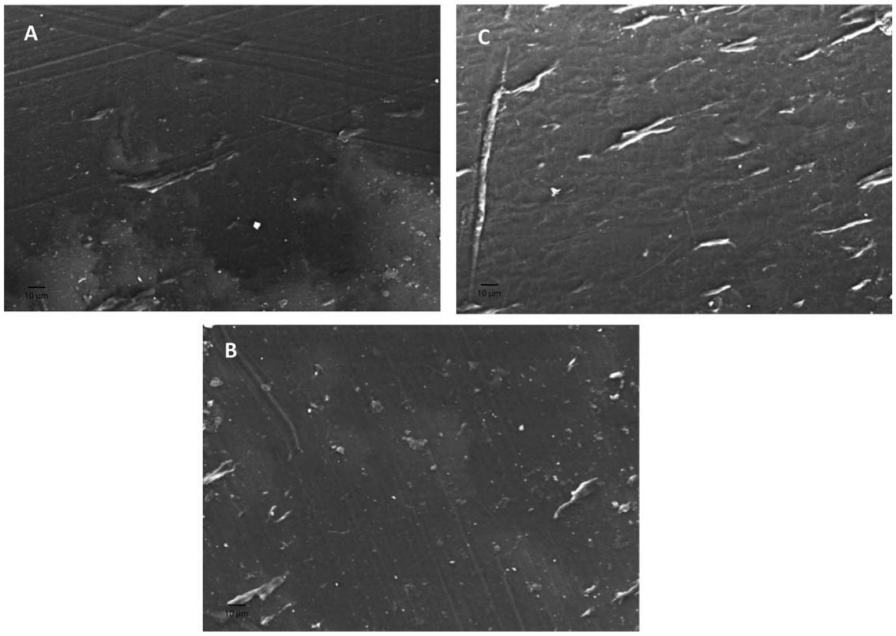


**FIGURE 5.** SEM results at magnification ×500 for different materials. A: Simple PVA. B: PVA loaded with PPy. C: PVA loaded with COOH- functionalized CNTs.

identified for PVA loaded with COOH- functionalized CNTs are similar of the bands detected for PVA. For PVA loaded with PPy new bands appeared at 1313 $cm^{-1}$ (C—N stretching vibration in the ring) and 1170 $cm^{-1}$ (C—H in-plane deformation).[14] Compared with PVA, the tube-guides of PVA loaded with COOH- functionalized CNTs and PVA loaded with PPy showed a less intensity of the peaks, especially between 2237 and 2380 $cm^{-1}$.

***X-ray diffraction (XRD) analysis.*** Figure 4 shows XRD patterns of the nerve tube-guides, and all indicate low crystallinity of the three biomaterials. The broad peak at 20° corresponds to a typical diffraction peak of PVA, and it can be observed in all tubes. Near 26° a broad scattering peak appears for the tube loaded with PPy, and is an indication of the presence of PPy as supported in literature.[21,22,24]

***Scanning electron microscope (SEM) analysis.*** SEM is an important technique in biomaterials to characterize the topography that is known to influence the cell response when associated cell-based therapies to the tube-guides or even for the axon, Schwann cells, fibroblasts, and inflammatory cells which play a crucial role during the Wallerian degeneration and nerve regeneration process. Figure 5 shows the morphology of the tube-guides studied. Both PVA and PVA loaded with PPy exhibited similar surface appearance [Fig. 5(A,B)]. The PVA loaded with COOH- functionalized CNTs showed a rough surface as expected due to the presence of CNTs on PVA matrix, with oriented features [Fig. 5(C)].

***Wettability analysis.*** Wettability was assessed by water contact angle measurements for all materials, and the results are presented in Table III. The contact angle measurements have been used to study changes in surface composition and migration of functional groups.[22] It is a very important surface property that influences the interaction of proteins/cells with a solid surface, once again, a crucial aspect during the Wallerian degeneration and nerve regeneration process. Results obtained with all materials showed a hydrophilic behavior

**TABLE III. Water Contact Angle for Different Nerve Guide Tubes**

| Sample | Contact angles (°) |
|---|---|
| PVA 15% (%w/v) | 45.7 ± 0.4 |
| PVA 15% loaded with PPy 0.05% (%w/v) | 66.2 ± 0.1 |
| PVA 15% loaded with COOH- CNTs 0.05% (%w/v) | 50.7 ± 0.4 |

**TABLE IV. Zeta Potential of Various Nerve Guide Tubes**

| Sample | Zeta Potential (mV) |
|---|---|
| PVA 15% (%w/v) | −4.97 ± 0.08 |
| PVA 15% loaded with PPy 0.05% (%w/v) | −1.17 ± 0.04 |
| PVA 15% loaded with COOH- CNTs 0.05% (%w/v) | −0.94 ± 0.02 |

**TABLE V. Summary of Mechanical Properties of Various Nerve Guide Tubes (*Statistically Different, $p < 0.05$)**

| Sample | Storage Modulus (Pa) | Tan σ |
|---|---|---|
| PVA 15% (%w/v) | $2.3 \times 10^4 \pm 7.8 \times 10^2$ | $7.8 \times 10^{-2} \pm 2.6 \times 10^{-3}$ |
| PVA 15% loaded with PPy 0.05% (%w/v) | $2.2 \times 10^4 \pm 9.5 \times 10^2$ | $8.0 \times 10^{-2} \pm 7.7 \times 10^{-3}$ |
| PVA 15% loaded with COOH- CNTs 0.05% (%w/v) | $1.7 \times 10^4 \pm 9.7 \times 10^2$ * | $8.2 \times 10^{-2} \pm 2.1 \times 10^{-3}$ |

(Table III). PVA presented a contact angle of $45.7° \pm 0.4°$, which is according to the bibliography.[23] The PVA loaded with PPy showed the highest value for the contact angle, and for that reason, it is considered the less hydrophilic nerve tube guide ($66.2° \pm 0.1°$). Literature reports for conductive PPy water contact angles around 80°,[24,25] and therefore the small reduction in wettability was expected. Once the graphitic carbon is reported as hydrophobic,[26] it was expected a higher contact angle for PVA loaded with COOH- functionalized CNTs compared to PVA tube-guide.

***Zeta potential analysis.*** The zeta potential results are presented at Table IV. All materials showed negative zeta potential being the PVA the most negative surface (−4.97 mV).

Zeta potential (ZP) is an indirect measurement of surface charge. It is a relevant parameter since it influences the interaction of the surface with proteins, and therefore affects the adhesion and growth pattern of cells. The value of simple PVA is consistent with the bibliography [23].

Zeta potential of CNTs is a function of its purity and functionalization. The values found in the literature for pristine MWCNTs vary widely from 0 mV to −29.3 mV, at pH = 7, probably due to different purities.[27] Therefore, and considering the low load level of CNTs, the more positive values of PVA loaded with COOH- functionalized CNTs are rather expected. Regarding the effect of PPy load on the zeta potential of PVA, since a value of $0.0 \pm 2.1$ mV[23] is reported in the literature to PPy, the value obtained of $-1.17 \pm 0.04$ mV compared to value of $-4.97 \pm 0.08$ mV of PVA is also expected.

***Dynamic mechanical analysis (DMA).*** The elastic and viscoelastic behavior of the developed nerve tube-guides was assessed by DMA. The storage modulus, which measures the material's elastic behavior, and the tan delta, which is a measure of the energy dissipation of a material were calculated. Experimental results obtained are summarized in Table V. The values of storage modulus were similar for PVA and PVA loaded with PPy and CNTs. However, the PVA loaded with COOH- functionalized CNTs showed a slightly lower modulus ($1.7 \times 10^4$ Pa). These results can be explained by the fact that PVA contains a large number of hydrophilic -OH groups, which can easily form hydrogen bonds with COOH- group of functionalized CNTs. Regarding the viscoelasticity, all the tested tube-guides showed statistically similar behavior (ANOVA, $p < 0.05$).

**Cytocompatibility using MSCs from the Whartoń s jelly**

Results obtained from epifluorescence technique confirmed that MSCs isolated from the Wharton's jelly of the umbilical cord did not begin the apoptosis process, showing that the MSCs are *in vitro* viable in the presence of the three biomaterials. The $[Ca^{2+}]_i$ measured was $43.8 \pm 6.5$ nM ($n = 15$), $48.2 \pm 3.6$ nM ($n = 15$), and $48.3 \pm 1.9$ nM ($n = 15$) for MSCs cultured in the presence of PVA, PVA 15% loaded with COOH- CNTs 0.05%, and PVA 15% loaded with PPy 0.05% discs after 7 days of culture, respectively. The MSCs cultured and expanded in the presence of the three tested biomaterials reached confluence and exhibited a normal star-like shape with a flat morphology in culture.

## DISCUSSION

This work focused on the development of electrical conductive nerve tube-guides, which was achieved by loading PVA with 0.05% of PPy or COOH-functionalized CNTs and their characterization prior to *in vivo* application in the rat sciatic nerve neurotmesis injury model. The inclusion of CNTs and PPy brought a significant increase of electrical conductivity of the simple PVA tube-guide.

The tube-guides with CNTs showed a rougher topography compared to the other ones, which is beneficial to associate this material to a cell-based therapy and to improve the cellular mechanisms associated to Wallerian degeneration and nerve regeneration process. The DSC and XRD studies revealed that all materials have low crystallinity, but similar crystallinity levels. The wettability studies indicated a hydrophilic behavior of all materials, being the PVA loaded with PPy slightly more hydrophobic. In terms of surface charge, the zeta potential measurements revealed that both COOH- functionalized CNTs and PPy loads turned the surface charge slightly more positive. Regarding the elastic behavior, the COOH- functionalized CNTs load induced a slight decrease of the rigidity of PVA.

According to the results obtained using the epifluorescence technique to evaluate the cytocompatibility of three of the developed biomaterials—PVA, PVA 15% loaded with COOH- CNTs 0.05%, and PVA 15% loaded with PPy 0.05%, it is reasonable to conclude that the three biomaterials are viable substrate for MSCs culture and survival and may be used in further *in vivo* trials.

From the results obtained up to now the novel nerve tube-guides seem to have clinical potential in peripheral nerve regeneration and in the near future, they should be included in preclinical trials, using the rat sciatic nerve model, in axonotmesis and neurotmesis standard injuries.[28,29]

## ACKNOWLEDGMENT

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## 2.2. Lesões de nervo periférico

### 2.2.1. Lesão de Axonotmese - *Evaluation of PVA biodegradable electric conductive membranes for nerve regeneration in axonotmesis injuries - the rat sciatic nerve animal model.*

O estudo de lesões de axonotmese permite a obtenção de resultados de forma mais consistente dado ser uma lesão subcrítica. A realização de ensaios *in vivo* recorrendo a lesões de axonotmese permitiram avaliar a *performance* dos biomateriais desenvolvidos e selecionar aqueles com maior potencial na regeneração de nervo periférico, em lesões de maior gravidade de neurotmese. Os resultados deste estudo encontram-se descritos no artigo científico submetido para publicação à revista *Journal of Biomedical Materials Research: Part A* a 18 de Abril de 2016:

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**Evaluation of PVA biodegradable electric conductive membranes for nerve regeneration in axonotmesis injuries - the rat sciatic nerve animal model.**

# Evaluation of PVA biodegradable electric conductive membranes for nerve regeneration in axonotmesis injuries – the rat sciatic nerve animal model.

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**Abstract**

The therapeutic effect of three polyvinyl alcohol (PVA) membranes loaded with electrically conductive materials - carbon nanotubes (*PVA-CNTs*) and polypyrrole (*PVA-PPy*) - were tested *in vivo* for neuro-muscular regeneration after an axonotmesis injury in the rat sciatic nerve. The membranes electrical conductivity measured was $1.5\pm0.5x10^{-6}$ S/m, $579\pm0.6x10^{-6}$ S/m, and $1837.5\pm0.7\ x10^{-6}$ S/m, respectively. At week-12, a residual motor and nociceptive deficit were present in all treated groups, but at week-12, a better recovery to normal gait pattern of the *PVA-CNTs* and *PVA-PPy* treated groups was observed. Morphometrical analysis demonstrated that *PVA-CNTs* group presented higher myelin thickness and lower g-ratio. The *tibialis anterior* (TA) muscle, in the *PVA-PPy and PVA-CNTs* groups showed a 9% and 19% increase of average fiber size area and a 5% and 10% increase of the "minimal Feret's diameter", respectively. No inflammation, degeneration, fibrosis or necrosis were detected in lung, liver, kidneys, spleen, and regional lymph nodes and absence of carbon deposits was confirmed with Von Kossa and Masson-Fontana stains. In conclusion, the membranes of *PVA-CNTs* and *PVA-PPy* are biocompatible and have electrical conductivity. The higher electrical conductivity measured in *PVA-CNTs* membrane might be responsible for the positive results on maturation of myelinated fibers.

**Keywords:** nerve regeneration, axonotmesis, PVA, nerve-guides, carbon nanotubes, polypyrrole, functional analysis, morphometric analysis, neurogenic atrophy.

## 1. Introduction

Peripheral nerve injury (PNI) has attracted public attention because of the societal and economic burden it has caused due to restriction of functional recovery of the individuals. Mechanical, thermal, chemical or ischemic factors can be responsible for peripheral nerve injuries which include secondary neurogenic muscle atrophy [1, 2]. The high costs associated to individuals' disability and morbidity motivated surgeons, clinicians and scientists to improve the current therapy and even increase the neuro-muscular regeneration. For this purpose, the development of biomaterials and cell therapies are crucial to therapeutic success. The sciatic nerve in rats is commonly used as a model for the study of peripheral nerve regeneration. When full peripheral nerve transection injuries (neurotmesis) occur, reconstructive surgical procedures are always necessary [3] but in case of axonotmesis injuries the surgical procedures is not necessary although the use of biomaterials or cellular therapies may increase the regeneration rate and diminish the neurogenic muscular atrophy responsible for functional disabilities [1, 4-6]. However this injury model is often used in pre-clinical validations of biomaterials or cell-based therapies since it is a very well-studied model [2, 5, 7-13]. The use of electrically conductive biomaterials to produce tube-guides is a promising therapeutic, especially when the neurotmesis injuries [9, 13, 14] have a gap which invalidates an end-to-end suture. The use of tube-guides will also allow the delivery of cell therapies in axonotmesis and neurotmesis injuries. Polyvinyl alcohol (PVA) is a polymer used as a biomaterial due to its biocompatibility, non-toxic, non-carcinogenic, swelling properties, and bio-adhesive characteristics and it can be used as host material in order to increase the solubility as well as the mechanical strength of conductive materials. It is also approved by Food and Drug Administration (FDA) [15, 16]. Poly(pyrrole) (PPy) and COOH-functionalized multiwall carbon nanotubes (MWCNTs) are two of the most studied electrical conductive materials for tissue engineering, including the peripheral nerve regeneration. Both

materials have unique properties such as conductivity [17] and can be incorporated in several synthetic biodegradable material, including the ones made of PVA matrix. In the present experimental work, the effect of three different polyvinyl alcohol (PVA) membranes in nerve regeneration after a standard axonotmesis lesion in the rat sciatic nerve was studied. The study included simple *PVA* membranes, and those produced with a matrix of PVA loaded with MWCNTs (*PVA-CNTs*) and PPy (*PVA-PPy*) [17]. The use of the sciatic nerve axonotmesis injury model was chosen for validation of the best PVA composition to be used in a more serious lesion of neurotmesis. The importance of those composites is based on the hypothesis that such composites can be used to host the growth of resident cells and that electrical stimulation can be applied directly to cells through the composite, which proved to be beneficial in many regenerative strategies

[18]. Many studies have demonstrated that the chemical composition of the local nerve injury environment is crucial for nerve regeneration including the presence of growth factors [19]. The association of biomaterials that not only have a positive effect on peripheral nerve regeneration but are also capable of carrying or supporting a cellular system responsible for orchestrating this environment, has been recently demonstrated by our research group [1, 5, 7-9, 11, 13, 20-23]. The morphological evaluation of the muscle *tibialis anterior* (TA) regeneration after the treatment of an axonotmesis injury with these three different membranes permitted the evaluation of neuromuscular recovery (and the neurogenic atrophy), and to correlate with the functional and morphological recoveries of the injured sciatic nerves [2]. The morphological assessment of the regenerated sciatic nerve and TA muscle was combined with functional evaluation during the healing period of 12 weeks which included the Withdrawal Reflex Latency (WRL) for the nociception function, the Extensor Postural Thrust (EPT) for determination of the motor deficit, the Sciatic Functional Index (SFI) and the Static Sciatic Index (SSI) and kinematic analysis of the ankle joint angle

at the four selected instants of the stance phase: Initial Contact (IC), Opposite Toe Off (OT), Heel Rise (HR) and Toe-Off (TO) [1, 2, 5, 7-9, 11, 20-22]. The TA muscle morphometric analysis was appropriate for the evaluation of nerve regeneration and subsequent muscle's reinnervation. The results obtained demonstrated the positive effect of using electrically conductive tube-guides, especially the *PVA-CNTs,* to promote nerve regeneration and limit the neurogenic atrophy of the regional innervated muscles usually observed after serious nerve injuries and prolonged healing periods.

## 2. Material and methods

### 2.1. Biomaterial membranes design and preparation

Synthetic biodegradable membranes of PVA (Aldrich, Mowiol 10-98), PVA loaded with COOH-functionalized multiwall carbon nanotubes MWCNTs (Nanothinx, NTX5, MWCNTs 97% -COOH) (*PVA-CNTs* membranes), and PVA loaded with PPy (Aldrich, 10-40 S/cm of conductivity) (*PVA-PPy* membranes) were prepared according to Gonçalves et al., 2016 and Ribeiro et al., 2015 [13, 24] Briefly, applying a casting technique using a silicone mould, a PVA aqueous solution 15% (w/v), a PVA aqueous solution 15% (w/v) supplemented with 0.05% of COOH-functionalized MWCNTs and a PVA aqueous solution 15% (w/v) supplemented with a 0.05% of PPy were prepared. The membranes were produced by freezing/thawing cycles as described in Ribeiro et al., 2015 and Gonçalves et al., 2016[13, 24]. Gamma-radiation was employed as sterilization method and the membranes were hydrated in a sterile saline solution during 2h before microsurgical application in the rat axonotmesis injuries (**Figure 1**). Membranes physical - chemical characterization included electrical conductivity assessment, Differential Scanning Calorimetry (DSC) and enthalpy determination (ΔH), Fourrier Transform Infrared Spectroscopy (FTIR), X-Ray Diffraction

(XRD), Scanning Electronic Microscopy (SEM) and wettability determination, and the results were published elsewhere [13, 24].

**2.2. Surgical procedure**

All the animal testing procedures were in conformity with the Directive 2010/63/EU of the European Parliament and with the approval of the Veterinary Authorities of Portugal in accordance with the European Communities Council Directive of November 1986 (86/609/EEC). Humane endpoints were always followed in accordance to the OECD Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation (2000). All surgeries were performed by a veterinary surgeon of the research team with Felasa – Category C accreditation for animal experimentation. Adult male Sasco Sprague Dawley rats (Charles River Laboratories, Barcelona, Spain) weighing approximately 250-300 g, divided in 4 groups of 7 animals each, were used. All animals were housed in a temperature and humidity controlled room with 12–12 hours light/dark cycles, 2 animals per cage (Polycarbonate cage type 3), and were allowed normal cage activities under standard laboratory conditions. The animals were fed with standard chow and water *ad libitum*. The anesthetic protocol used and the surgery techniques performed were the ones described previously elsewhere [25-27]. A standardized crush injury was performed (axonotmesis) using a non-serrated clamp (manufactured by the Institute of Industrial Electronic and Material Sciences, University of technology, Vienna, Austria) exerting a constant force of 54 N, for a 30 seconds period to create a 3 mm long crush injury, 10 mm above the bifurcation into tibial and common peroneal nerves. The starting diameter of the sciatic nerve was about 1 mm, flattening during

the crush to 2 mm [25-27] (**Figure 2**). Four experimental groups were studied. In Groups 1 to 3 the sciatic nerve crush injury was wrapped by membranes: in Group 1 the crush nerve was wrapped with a PVA membrane (Group 1, *PVA*), in Group 2, the crushed nerve was wrapped with a PVA membrane loaded with 0.05% (%w/v) of COOH-functionalized MWCNTs (Group 2, *PVA-CNTs*), in Group 3 the crushed nerve was wrapped with a PVA membrane loaded with Ppy 0,05% (%w/v) (Group 3, *PVA-PPy*) and in Group 4 the axonotmesis was performed without any additional procedure (Group 4, *Control*) (**Figure 2**).

### 2.3. Functional assessment

After standardized axonotmesis injury and sciatic nerve microsurgical reconstruction using the developed scaffolds, a follow-up consisting of the measurement of functional parameters important to evaluate the regeneration process during the healing period was performed. Animals have been tested pre-operatively (week-0), every week until week-8, and then every 2 weeks until the end of follow-up time (12 weeks). The ankle kinematics analysis was carried out prior nerve injury, and at week-12 of follow-up time, in the following experimental groups: *PVA*, *PVA-CNTs*, *PVA-PPy* and *Control*. Animals were gently handled, and tested in a quiet environment to minimize stress.

#### 2.3.1. Motor performance and nociceptive function.

Motor performance and nociceptive function were evaluated by measuring extensor postural thrust (EPT) and withdrawal reflex latency (WRL), respectively. To assess the nociceptive withdrawal reflex (WRL), the hotplate test was modified as described by Masters and collaborators [28]. The animal is wrapped in a surgical towel above its waist and then positioned to stand with the affected hind paw on a hot plate, at 56ºC, and with the other on a room temperature plate. WRL is defined as the time elapsed from the onset of hotplate

contact to withdrawal of the hind paw and measured with a stopwatch. Normal animals withdraw their paws from the hotplate within 4.3 s or less [4, 8, 11, 22, 29, 30]. The affected limbs were tested 3 times, with an interval of 2 min between consecutive tests to prevent sensitization, and the three latencies were averaged to obtain a final result [31]. If there was no paw withdrawal after 12 s, the heat stimulus was removed to prevent tissue damage, and the animal was assigned the maximal WRL of 12 s[25, 32].

The EPT was originally proposed by Tralhammer et al [33][34] as a part of the neurological recovery evaluation in the rat after sciatic nerve injury. For this test, the entire body of the animal, with exception of the hind-limbs, is wrapped in a surgical towel. EPT is induced by supporting the animal by the thorax and lowering the affected hind-limb towards the platform of a digital balance. As the animal is lowered to the platform, it extends the hind-limb, anticipating the contact made by the distal metatarsus and digits. The force in grams (g) applied to the digital platform balance is recorded. The same procedure is applied to the contra-lateral, unaffected limb. For the EPT test, the affected and normal limbs are tested 3 times, with an interval of 2 minutes between consecutive tests, and the 3 values are averaged to obtain a final result. The normal (unaffected limb) EPT (NEPT) and experimental EPT (EEPT) values are incorporated into an equation (**Equation 1**) to derive the percentage of functional deficit, as described in the literature[4, 8, 21, 30, 33, 34].

$$\% \text{ Motor deficit} = [(\text{NEPT} - \text{EEPT}) / \text{NEPT}] \times 100 \quad \textbf{(Equation 1)}$$

**2.3.2. Sciatic Functional Index (SFI) and Static Sciatic Index (SSI).**

For Sciatic Functional Index (SFI), animals are usually tested in a confined walkway that they cross, measuring 42 cm long and 8.2 cm wide, with a dark shelter at the end. Several measurements must be taken from the footprints: i) distance from the heel to the third toe, the print length (PL); ii) distance from the first to the fifth toe, the toe spread (TS); and iii)

distance from the second to the fourth toe, the intermediary toe spread (ITS). In the static sciatic index evaluation (SSI) only the parameters TS and ITS, are measured. For SFI and SSI, all measurements are taken from the experimental (E) and normal (N) sides. Prints for measurements are chosen at the time of walking based on precise, clear and completeness of footprints. The mean distances of three measurements are used to calculate the following factors (dynamic and static): SFI is calculated as described by Bain et al. (1989) [35] according to the following Equation 2:

$$SFI = -38.3(EPL - NPL) / NPL + 109.5(ETS - NTS) / NTS + 13.3(EIT - NIT) / NIT - 8.8$$

$$= (-38.3 \times PLF) + (109.5 \times TSF) + (13.3 \times ITSF) - 8.8 \quad \textbf{(Equation 2)}$$

SSI is calculated as described by Bervar et al [36] according to the following Equation 3:

$$SSI = 108.4 \text{ x } TSF + 31.85 \text{ x } ITF - 5.49 \quad \textbf{(Equation 3)}$$

For SFI and SSI, an index score of 0 is considered normal and an index of -100 indicates total impairment. When no footprints are measurable, the index score of -100 is given. In each walking track 3 footprints should be analyzed by a single observer, and the average of the measurements is used in SFI calculations.

### 2.3.3. Kinematic analysis

Ankle kinematics analysis was carried out prior nerve injury, and at week-12 of follow-up time, in the following experimental groups: *PVA*, *PVA-CNTs*, *PVA-PPy* and *Control*. Animals walked on a Perspex track with length, width and height of 120, 12, and 15 cm, respectively. In order to ensure locomotion in a straight line, the width of the apparatus was adjusted to the actual animal size during the experiments. The animals' gait was video recorded at a rate of 300 Hz images per second (Casio Exilim PRO EX-F1, Japan). The camera was positioned at the track half-length where gait velocity was steady, and 1 m distant

from the track obtaining a visualization field of 14 cm wide. The video images were stored in a computer hard disk for latter analysis using an appropriate software APAS® (Ariel Performance Analysis System, Ariel Dynamics, San Diego, USA). 2D biomechanical analyses (sagittal plane) was carried out applying a two-segment model of the ankle joint, adopted from the model first developed by [37, 38]. The animals' ankle angle was determined using the scalar product between a vector representing the foot and a vector representing the lower leg. With this model, positive and negative values of position of the ankle joint (Θº) indicate dorsiflexion and plantarflexion, respectively. For each step cycle the following time points were identified: initial contact (IC), Opposite Toe off (OT), and Heel Rise (HR) and Toe-off (TO) [22, 32, 38] and were time normalized for 100% of step cycle. The normalized temporal parameters were averaged over all recorded trials. A total of 6 walking trials for each animal with stance phases lasting between 150 and 400 ms were considered for analysis, since this corresponds to the animal's normal walking velocity (20–60 cm/s) [22, 37, 38].

### 2.4. Morphological analysis and histopathology

#### 2.4.1. Morphological assessment of nerve

Nerve samples (10-mm-long sciatic nerve segments distal to the crush site and from un-operated controls) were processed for quantitative morphometry of myelinated nerve fibers. Fixation was carried out using 2.5% purified glutaraldehyde and 0.5% saccarose in 0.1M Sorensen phosphate buffer for 6-8 hours and resin embedding was obtained following Glauerts' procedure. Series of 2-µm thick semi-thin transverse sections were cut using a Leica Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) and stained by Toluidine blue. Stereology was carried out on a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany). Systematic random sampling and D-disector were adopted using a protocol

previously described [39, 40]. Total number of myelinated fibers, axon size, myelin thickness and g-ratio were evaluated.

**2.4.2. Morphological assessment of muscle**

At termination of the functional testing performed in this study, *tibialis anterior* (TA) muscles of all the axonotmesis and TA muscles without lesion (*Pre crush* group) were collected, and the tissue samples were fixed in 10% buffered formalin, routinely processed, dehydrated and embedded in paraffin wax. Consecutive 3 µm transverse sections from the mid-belly of each muscle were cut and stained with haematoxylin and eosin (HE) and kept for morphometry and determination of degree of atrophy. For the morphometric analysis, an unbiased sampling procedure was applied. The muscle fibers' cross section area and "minimal Feret's diameter" which is the minimum distance of parallel tangents at opposing borders of the muscle fiber, were evaluated from the cross sections using the ImageJ© software (NIH) which allowed to apply this set of individual fiber measurements. A minimum of 1000 skeletal muscle fibers was measured from each group. This assessment was performed by 2 independent operators. Each one of the operators blindly and randomly measured an average of 50 fibers in each section. Images were acquired using a Nikon® microscope connected to a Nikon® digital camera DXM1200, at low magnification (100x) under the same conditions that were used to acquire a reference ruler.

**2.4.3. Histology of internal organs**

At the end of the study, all animals were subjected to a complete necropsy examination in order to evaluate the presence of possible internal anomalies and/or injuries. Spleen, liver,

kidney, lung and lymph nodes were collected, weighed and submitted to histological examination to identify putative related microscopic alterations such as inflammation, degeneration, or accumulation of biological material. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Three consecutive sections of 3 µm were made, one being stained with hematoxylin-eosin (HE) and the others were used for histochemical staining. Microscopically, massive carbon deposits generally appear as well-recognized anthracotic black pigment, especially in the lung. A recent report described that some types of carbon nanotubes may appear as small punctuated intracellular accumulations in the Kuepfer cells (liver) and in the intermediate zone of the spleen, when intravenously administered [41]. Special stains such as Von Kossa (VK - method that demonstrates phosphates and carbonates) and Masson-Fontana (MF - method used for distinguish carbon deposits from melanin) were performed in order to exclude the presence of these substances in the different tissues under study concerning the experimental group of *PVA-CNTs*.

**2.4 Statistical analysis**

For sciatic nerve functional analysis, a mixed model repeated measures ANOVA was used to test for differences across time and sciatic nerve treatment. Sphericity was assessed by Mauchly's test and Greenhouse-Geisser degrees of freedom correction was used in cases sphericity could not be assumed or when corrected p-values were below the accepted level of significance ($P < 0.05$). Tukey's HSD test was used for pairwise comparisons. Ankle kinematics data were analyzed with One-way ANOVA. All data is presented as mean and standard deviation (SD), unless otherwise stated. These statistical tests were carried out with IBM SPSS Statistics Version 19. For stereology, statistical comparisons of quantitative data were subjected to one-way ANOVA test, followed by pairwise comparisons using Tukey's HSD test. Statistical significance was established as $P<0.05$. Stereological data was analyzed

using the software using the SPSS version 19.0 (SPSS, Chicago, IL). For muscle morphometry, statistical analysis was performed using the SPSS version 19.0 (SPSS, Chicago, IL). Results are presented as mean ± SEM in Figure 5 and Figure 6. Multiple comparisons between groups were performed by one-way ANOVA supplemented with Tukey's HSD post hoc test. Differences were considered statistically significant at $P < 0.05$.

## 3. Results

### 3.1. Biomaterial characterization

The three different tube-guides of PVA were physicochemical characterized (*PVA*, *PVA-CNTs* and *PVA-PPY*). All the results were in agreement with the findings previously reported [13, 24]. The electrical conductivity achieved for the three different membranes (*PVA*, *PVA-CNTs* and *PVA-PPy*) was 1.5±0.5x$10^{-6}$ S/m, 579±0.6x$10^{-6}$ S/m, and 1837.5±0.7 x$10^{-6}$ S/m, respectively **(Table 1)**. In light of the conductivity results, the three membranes (simple PVA, PVA loaded with 0.05% (%w/v) of COOH-functionalized CNTs and PPy) compositions were chosen for *in vivo* application in the rat sciatic nerve axonotmesis and neurotmesis injury models [13, 24]. The thermal characteristics of simple PVA and loaded PVA materials (*PVA-CNTs* and *PVA-PPY*) were examined by Differential Scanning Calorimetry (DSC) and Enthalpy of Fusion (ΔH) was calculated, and the percentage of crystallinity was near 7.4% for all analyzed nerve membranes. Fourier Transform Infrared Spectroscopy (FTIR) analysis of the bands identified for PVA loaded with COOH-functionalized CNTs (*PVA-CNTs*) were similar to the bands detected for simple *PVA*. For PVA loaded with PPy (*PVA-PPy*) new bands appeared at 1313 $cm^{-1}$ (C-N stretching vibration in the ring) and 1170 $cm^{-1}$ (C-H in-plane deformation). Compared with simple *PVA,* the other membranes showed inferior intensity of the peaks, especially between 2237 and 2380 $cm^{-1}$[24] [13]. Considering the X-ray Diffraction (XRD) analysis previously performed, the broad peak observed at 20º corresponded to a typical diffraction peak of *PVA*, and it could be also observed in all

membranes. Near 26º a broad scattering peak appeared for the tube loaded with PPy (*PVA-PPy*), and it was an indication of the presence of PPy as supported in literature[13, 42]. Both *PVA* and PVA loaded with PPy (*PVA-PPy*) when analyzed by Scanning Electron Microscope (SEM) exhibited similar surface appearance. On the other hand, the PVA loaded with COOH-functionalized CNTs (*PVA-CNTs*) showed a rougher surface as expected due to the presence of CNTs on PVA matrix, with oriented features [13] [24]. This characteristic was determinant on the choice of this biomaterial to be associated to the MSCs (*PVA-CNTs-MSCs* group) in sciatic nerve neurotmesis injury reconstruction with functional and morphologic positive regenerating outcomes [13, 43]. The wettability analysis showed a hydrophilic behavior for the three biomaterials used for tube-guide tested, also the three materials showed negative zeta potential being the *PVA* the most negative surface (-4.97 mV) [13] [24] (**Table 1, Figure 1**).

### 3.2. Functional assessments of re-innervation

Immediately after axonotmesis, the crushed areas of all sciatic nerves were flattened but nerve continuity was preserved. Following crush injury, a complete sciatic nerve paralysis was observed in all experimental animals. All animals survived without auto-mutilation and normal wound healing. All rats were evaluated pre-operatively (week-0) and every week during 8 weeks and then at week-10 and week-12, when the animals were sacrificed for morphological analysis of nerve and TA muscles.

#### 3.2.1. Motor deficit and nociceptive function

##### 3.2.1.1. Motor deficit - Extensor Postural Thrust (EPT)

Values of percentage of functional deficit (%), were obtained by performing the extensor postural thrust (EPT) test, preoperatively (week-0), and every week during 8 weeks and then

at weeks 10 and 12 **(Figure 3)**. In the week following sciatic nerve crush, motor deficit was severe and compatible with almost complete loss of postural extensor thrust response in the affected side **(Figure 3)**. The mixed two-way ANOVA results indicate a significant effect of time for motor deficit values [$F_{(9,216)}$ = 22.379; P < 0.001] but no time *versus* group interaction or group effects. The simple contrasts showed that significant improvement in motor deficit could be noticed from week-3 post-injury onwards. One-way ANOVA showed that no differences between the groups existed in motor deficits scores before sciatic nerve crush. At week-12, the three experimental groups (*PVA*, *PVA-CNTs*, *PVA-PPy*) presented a residual motor deficit of 0.16±0.31, 0.10±0.41, and 0.16±0.59 respectively, The individual variability between animals of the same experimental group is evidenced due to the subjectivity of the test, but it can be concluded that no differences between the experimental groups existed in motor deficits scores at week-12 sciatic nerve crush by one-way ANOVA **(Figure 3)**.

**3.2.1.2. Nociception function – Withdrawal Reflex Latency (WRL)**

Values in seconds were obtained by performing the WRL test, preoperatively (week-0), and every week during 8 weeks and then at weeks 10 and 12. A severe loss of thermal sensitivity in the affected paw could be noticed immediately after sciatic nerve crush **(Figure 4)**. From such acute thermal and pain sensitivity loss, a steady recovery of the withdrawal response to thermal stimulus occurred throughout the following weeks such as mixed two-way ANOVA could found a significant effect of time [$F_{(9.216)}$ = 6.072; P < 0.001] but no time *versus* group interaction or group effects. According to simple contrasts testing, significant improvement in the latency for paw withdrawal took place following week-4. One-way ANOVA showed that no differences between the groups existed in withdrawal reflex latency times before sciatic nerve crush. At week-12, the three experimental groups (*PVA*, *PVA-CNTs*, *PVA-PPy*)

presented a withdrawal response to thermal stimulus near normal values of 4.3s (5.26±0.15, 5.34±1.43, and 5.28±1.43 respectively). The individual variability between animals of the same experimental group is evidenced once again by the subjectivity of the test which emphasizes the need to perform kinematics analysis of the gait of the treated rats. It can be concluded that no differences between the experimental groups existed considering the nociceptive function scores at wek-12 sciatic nerve crush by one-way ANOVA **(Figure 4)**.

**3.2.2. Sciatic Functional Index (SFI) and Static Sciatic Index (SSI)**.

Scores of SSI drop severely immediately after sciatic nerve crush and thereafter showed a progressive recovery **(Figure 5)**. Therefore, two-way ANOVA found a significant time effect [$F_{(9.189}$ = 19.218; P < 0.001] but once again without time *versus* group interaction and group effects. The recovery of SSI scores started to be consistently significant from week-5. One-way ANOVA showed that no differences between the groups existed in SSI scores before sciatic nerve crush. At week-12, the three experimental groups (*PVA*, *PVA-CNTs*, *PVA-PPy*) presented SSI scores of -8.81±37.50, -14.79±20.65, and -19.80±12.39 respectively **(Figure 5)**.

Scores of SFI measured in the week following sciatic nerve crush reached very low values, indicating severe loss of sciatic nerve function **(Figure 6)**. From this time point, SFI scores showed a very robust recovery so that two-way ANOVA found a significant time effect [$F_{(9.189}$ = 76.708; P<0.001], with recovery starting as soon as week-2. Again, no time versus group and group effects existed. Likewise, no differences between the groups could be found before sciatic nerve crush. At week-12, the three experimental groups (*PVA*, *PVA-CNTs*, *PVA-PPy*) presented SFI scores of -14.01±10.40, -12.55±5.57, and -12.86±11.56 respectively **(Figure 6)**.

### 3.2.3. Kinematic analysis

For kinematic analysis, measures of ankle joint angle at the four selected instants of the stance phase (IC, OT, HR and TO) were collected before sciatic nerve injury and at the end of 12-weeks recovery period (N = 7) **(Table 1, Figure 7)**. At 12-weeks post-injury, ankle kinematics improved in every group approaching its pre-injury pattern. At this time point, ankle angle was similar at IC and it was similar for every group of animals. However, at OT and HR subtle differences between groups in ankle angle could be devised, with animals treated with conductive membranes revealing slightly abnormal ankle kinematics, comparing with the control and uninjured animals ($P < 0.05$). At TO normal ankle angle values were registered for all groups with the relative exception of the PVA group for which the ankle seemed somewhat excessive at this instant of the gait cycle, particularly when comparing with uninjured animals ($P = 0.065$).

### 3.3. Morphological analysis and histopathology

#### 3.3.1. Histomorphometry of the regenerated sciatic nerve

Histological analysis on Toluidine Blue stained semithin sections showed that nerve fiber regeneration occurred in all repaired nerves 12 weeks after surgery **(Figure 8A-C)** with a micro-fasciculation typical of regenerated nerve fibers. Morphometrical analysis **(Figure 8D)** allowed to compare the three groups in terms of number of myelinated fibers, axon diameter and myelin thickness. Moreover, g-ratio was also calculated in order to better appreciate maturation of myelinated fibers. The *PVA* group (N = 7), presented a total number of myelinated fibers of 16596±1737, axon diameter (d) of 2.95±0.17, fiber diameter (D) of 3.56±0.21, myelin thickness (M) of 0.31±0.02, ratio axon diameter and fiber diameter (d/D, g-ratio) of 0.82±0.01. The *PVA-CNTs* regenerated nerves (N = 7) presented a total number of myelinated fibers of 16049±2442, axon diameter (d) of 2.98±0.18, fiber diameter (D) of

3.71±0.20, myelin thickness (M) of 0.37±0.02, ratio axon diameter and fiber diameter (d/D, g-ratio) of 0.79±0.01. The *PVA-PPY* regenerated nerves (N=7) presented a total number of myelinated fibers of 22588±3596, axon diameter (d) of 3.03±0.10, fiber diameter (D) of 3.70±0.12, myelin thickness (M) of 0.33±0.01, ratio axon diameter and fiber diameter (d/D, g-ratio) of 0.81±0.00 **(Figure 8D)**. The results described showed no differences in terms of number of myelinated fibers and axon diameter between the three groups, but it's possible to appreciate statistically significant differences between *PVA* and *PVA-CNTs* groups in the evaluation of myelin thickness (*P ≤ 0,05) and g-ratio (**P ≤ 0,01) and between *PVA-PPY* and *PVA-CNTs* as regards g-ratio (d/D) evaluation (*P ≤ 0,05). The decrease of g-ratio when axon diameter is similar, is an index of a better maturation of myelinated fibers **(Figure 8D)**.

### 3.3.2. Morphological analyses of the *tibialis anterior* (TA) muscle

Following 12 weeks of sciatic nerve crush it was possible to observe by muscle morphometry that there was a significant difference ($P < 0.05$) in terms of fiber size between sciatic nerve without lesion (*Pre crush*), sciatic nerve with axonotmesis injury without surgical reconstruction (*Control*) and the treatment (*PVA, PVA-PPy and PVA-CNTs*) groups. In the *PVA-PPy* treatment group there was a 9% increase in terms of average fiber size area and a 5% increase in term of the "minimal Feret's diameter", when comparing to the untreated *Control* group **(Figure 9)**. In the *PVA-CNTs* treatment group there was a 19% increase in terms of average fiber size area and a 10% increase in term of the "minimal Feret's diameter", when comparing to the untreated *Control* group. Opposite results were observed for the *PVA* group in which the mean fiber size area and "minimal Feret's diameter" were significantly lower than the untreated group (17% and 9% respectively) (**Figure 9**).

### 3.3.3. Histology of internal organs

At the histological examination, no alterations compatible with images of inflammation, degeneration, fibrosis or necrosis were detected in all animals from the three experimental groups (*PVA*, *PVA-PPy* and *PVA-CNTs*). The negative results obtained with both Von Kossa and Masson-Fontana stains reinforced the absence of nanotubes and carbon deposits in all the organs in all animals from the experimental group of *PVA-CNTs* (**Figure 10**).



**4. Discussion**

The peripheral nervous system (PNS) establishes the connection between the central nervous system (CNS) and the peripheral structures, including the innervating of regional skeletal muscles. Innervation regulates skeletal muscle mass and muscle phenotype and changes in the muscles may contribute to functional deficit after nerve injury [2]. The neuromuscular regeneration analysis is very important to evaluate new therapeutic approaches that will allow better functional recovery of the individuals, sometimes limited by the regional muscle atrophy and fibrosis. After injury, the functional recovery can be partially gained in the PNS because of the presence of Schwann cells (SCs), which are able to supply nutrient support, guide and myelinate regenerating axons, and provide growth factors [5], where the Wallerian degeneration has a crucial role [2, 5]. After axonotmesis, spontaneous regeneration through the distal nerve stump can be expected but a total functional recovery is frequently not achieved. By reducing the healing period after axonotmesis, using several therapeutic strategies, and promoting the peripheral nerve regeneration, the secondary neurogenic muscle atrophy can be diminished, with improvement of the functional outcome [2, 5]. Experiments on peripheral nerve regeneration are often performed on the rat sciatic nerve model [44] and the axonotmesis injury, on the other hand, is the most widely used *in vivo* model to test

several biomaterials for tube-guide nerve fabrication and more recently, for cellular therapies that are applied in more serious nerve injuries like the neurotmesis [2][4, 7-13, 29][45].

In the present experimental work, it was studied the effect of three different PVA membranes in nerve regeneration after a standard axonotmesis lesion in the rat sciatic nerve: simple *PVA*, and those produced with a matrix of PVA loaded with MWCNTs (*PVA-CNTs*) and PPy (*PVA-PPy*). These last two types of membranes presented increased electrical conductivity when compared with the PVA membrane, which was considered as being an advantage for nerve regeneration [17]. PVA is a biodegradable, water-soluble synthetic polymer that has been increasingly used in biomedical applications. PVA may mimic the regulatory characteristics of natural extracellular matrix (ECM) and ECM-bound growth factors [9]. Its hemocompatibility and biocompatibility was previously confirmed when PVA was previously used by our research group in vascular grafting [46]. PVA was also previously evaluated in association with human MSCs (hMSCs) isolated from the umbilical cord (UC) Wharton's jelly. For this purpose hMSCs were cultured on PVA membranes and it was proved the cytocompatibility and biointegration of the developed biomaterial associated to this cellular system when used *in vivo* in pre-clinical trials in ovine animal model [46] and rat neurotmesis sciatic nerve injury model [13]. The polymers with electrical conductivity have attracted interest because they simultaneously display the physical and chemical properties of organic polymers and the electrical characteristics of metals which might improve the neuromuscular regeneration. The importance of those polymers is based on the hypothesis that such biomaterials can be used to host the growth of cells, and electrical stimulation can be applied directly to the cells, which proved to be beneficial in many regenerative strategies, where neuromuscular regeneration is the one of the most promising areas [9] [17][47]. The three different tube-guides / membranes of PVA (*PVA*, *PVA-CNTs* and *PVA-PPY*) were physicochemical characterized prior to *in vivo* application. As a matter of fact, PVA with

CNTs or PPy resulted in conductive biomaterials with higher electrical conductivity than the polymer matrix, and all the results were in agreement with the findings previously reported for such materials [13, 24]. The electrical conductivity achieved for the three different membranes (*PVA*, *PVA-CNTs* and *PVA-PPy*) was 1.5±0.5x$10^{-6}$ S/m, 579±0.6x$10^{-6}$ S/m, and 1837.5±0.7 x$10^{-6}$ S/m, respectively **(Table 1),** demonstrating that the electrical conductivity of *PVA-PPy* and *PVA-CNTs* are around 500 and 2000 times greater than the one observed to *PVA* alone, respectively [24].

It is crucial to combine both neuromuscular functional and morphological assessment, which was performed in the present described *in vivo* trials. It is not generally agreed which type of evaluation tool is the most useful descriptor of functional recovery; for this reason, the use of different methods for an overall assessment of nerve function has been recommended by several investigators [48] and it is applied in the present experimental work, including WRL and EPT functional tests, SFI, SSI and kinematic analysis of the gait and morphometric analysis of the regenerated sciatic nerves and TA muscles. Among the wide range of available tests, EPT, SFI, SSI and WRL, proved to be reliable, valid and efficient methods to determine functional recovery after sciatic nerve injury in previous studies but with some subjectivity in the results analysis [30][5], so it is important to include the gait kinematic analysis and quantitative morphological evaluation of the regenerated nerve and TA muscle [2, 5]. Indeed, the use of biomechanical parameters has given valuable insight into the effects of the sciatic nerve denervation/reinnervation, and thus represents an integration of the neural control acting on the ankle and foot muscles, which is very useful and accurate to evaluate different therapeutic approaches [25, 37, 48]. It is important to realize that the number of kinematic variables (positions, velocities, and accelerations) required to describe one-step cycle is very high. Therefore, it is only through high speed digital cameras that we can achieve a full kinematic description during gait [49]. Significant improvement in motor

deficit could be noticed from week-3 post-injury onwards and at week-12, a residual motor and nociceptive deficit were present in the three treated groups, with no statistic differences. Also, the SSI and SFI scores were not different between the three experimental groups, showing a more pronounced recovery at week-5 and week-2, respectively. At 12 weeks post-injury ankle kinematics improved in every group and at TO, normal ankle angle values were registered for all groups with the relative exception of the *PVA* group, demonstrating a better recovery to normal gait pattern in animals treated with *PVA-CNTs* and *PVA-PPy*. Anyway, one should bear in mind that individual joint kinematics either in control or nerve-injured animals is characterized by high variability, with notable differences between different animals and even from step to step [50]. Such high level of variability, which seems to be an intrinsic property of normal quadruped walking, might affect in some degree the precision of joint kinematic measures of functional recovery after nerve injury [21].

The regenerated nerves and TA muscles were processed for morphology studies after the healing period of 12 weeks that included quantitative morphometry of myelinated nerve fibers and muscle fibers' cross section area and "minimal Feret's diameter" quantifications. Considering the morphometric evaluation of the TA muscle, in the *PVA-PPy and PVA-CNTs* treatment groups there were a 9% and 19% increase in terms of average fiber size area and a 5% and 10% increase in term of the "minimal Feret's diameter", respectively, when comparing to the untreated *Control* group. It is also interesting to observe that not only the two most promising treatment groups (*PVA-PPy* and *PVA-CNTs*), but also the sham (*Control*) exhibited better scores than *Pre crush* (no lesion) group. These results should be explained by the increased nerve sprouting in the nerves submitted to the crush lesion which might have produced a significant effect in terms of promotion of the muscle' re-innervation. Similar results were also described in a previous work on *tibial* nerve crush injury, in which hyper-innervations of neuromuscular junctions (NMJs) were observed after injury and

reverted back to the normal level at the same time as the functional recovery [51]. The recent published paper by di Summa et al. [52], where collagen nerve tube-guides (Neurogen®) were used *in vivo* to promote peripheral nerve regeneration, combined with SCs, it was possible to demonstrate an improved distal stump sprouting. The sprouting was more pronounced in the experimental group where the SCs were derived from bone marrow mesenchymal stem cells (BM-MSCs) when compared to SCs derived from adipose tissue mesenchymal stem cells (AT-MSCs). On the other hand, no significant differences were observed in proximal regeneration among all the experimental groups. BM-MSCs and AT-MSCs -loaded conduits showed a diffuse sprouting pattern, while loaded SCs showed an enhanced cone pattern and a typical sprouting along the conduits walls, suggesting an increased affinity for the collagen type I fibrillar structure. This observation is important and should be related to results obtained from the innervated muscle morphometry analysis. It should be kept in mind that the sprouting is also evaluated and a constant observation in the histomorphometry analysis of the regenerated peripheral nerve after axonotmesis and neurotmesis lesions, where different reconstruction strategies were tested *in vivo* in the rat sciatic nerve model by our research group for the past years [2, 4, 7-13, 24, 29, 30]. Morphometrical analysis of the regenerated nerves showed no differences in terms of number of myelinated fibers and axon diameter between the three treated groups, but it is possible to appreciate statistically significant differences between *PVA* and *PVA-CNTs* groups in the evaluation of myelin thickness and g-ratio, and between *PVA-PPY* and *PVA-CNTs* as regards g-ratio evaluation. The *PVA-CNTs* group presented higher myelin thickness and lower g-ratio which proves a better maturation of myelinated fibers.

The histopathology of lung, liver, kidneys, and regional lymph nodes was performed to ensure the biocompatibility of these biomaterials, mostly what concerns the possible concentration of CNTs in these organs after biodegradation. No alterations compatible with

images of inflammation, degeneration, fibrosis or necrosis were detected and the negative results obtained with both Von Kossa and Masson-Fontana stains reinforced the absence of nanotubes and carbon deposits in all the analyzed organs. These results were in agreement with previous *in vitro* experiments using hMSCs from the umbilical cord matrix that proved the cytocompatibility of these three biomaterials [13, 24]. Also the biocompatibility of these biomaterials was already previously proven in the ovine model when it was used as a vascular prosthesis [46], but also in the rat sciatic nerve neurotmesis injury model [13].

In conclusion, the membranes of *PVA-CNTs* and *PVA-PPy* are biocompatible and have electrical conductivity, which was benefit to nerve regeneration in terms of neuromuscular morphological regeneration but also from functional recovery point of view. The higher electrical conductivity measured in *PVA-CNTs* membrane might be responsible for the best results obtained in terms of peripheral nerve regeneration, demonstrating higher myelin thickness and lower g-ratio which proves a better maturation of myelinated fibers and it is also in line with the trend observed in results of kinematics analysis. Also, the neurogenic atrophy of the TA muscle was less significant in the rats where the crushed sciatic nerve was enwrapped in *PVA-CNTs* membranes, since better results in terms of average fiber size area and "minimal Feret's diameter" was observed. The results obtained sustain that *PVA-CNTs* membranes promote neuromuscular regeneration, by improving functional recovery and myelination of the regenerated nerve fibers. In this way, the neurogenic atrophy of the TA muscle is not so pronounced due to the associated reduced healing period.



## 5. Acknowledgements

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Programa Operacional Factores de Competitividade, Projects Pest-OE/AGR/UI0211/2011 and UID/CVT/00772/2013 funding from FCT. AR Caseiro would also like to acknowledge the FCT for the PhD grant SFRH/BD/101174/2014. IPATIMUP integrates the i3S Research Unit, which is partially supported by FCT. This work was funded by FEDER funds through the Operational Programme for Competitiveness Factors - COMPETE and National Funds through the FCT, under the project number PEst-C/SAU/LA0003/2013.

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## 7. Legends of Figures and Tables

**Figure 1:** Synthetic biodegradable tube-guides / membranes of *PVA* (1), PVA loaded with COOH-functionalized multiwall carbon nanotubes MWCNTs (*PVA-CNTs*) (2), and PVA loaded with PPy (*PVA-PPy*) (3), used to enwrapped the sciatic nerve with a standardized axonotmesis injury **(A)**. Schematic representation of the freezing/thawing process and the annealing treatment used for *PVA*, *PVA-CNTs*, and *PVA-PPy* membranes preparation **(B)**.

**Figure 2:** Photographs of the surgery procedure. Incision in the skin from the greater trochanter to the mid-half distally followed by a muscle splitting incision **(A)** and the right sciatic nerve was exposed **(B)**. After sciatic nerve mobilization, a standardized crush injury was performed (axonotmesis) using a non-serrated clamp **(C, D)**. The sciatic nerve crush injury was wrapped by different PVA electrical conductive materials **(E)**. Skin suture **(F)**.

**Figure 3:** Graphical representation of the mean values of Motor Deficit obtained performing Extensor Postural Thrust (EPT) test. This test has been performed preoperatively (week-0), and every week during 8 weeks and then at week-10 and week-12. Results are presented as mean and standard error of the mean (SEM). N corresponds to the number of rats within the experimental group (N = 7).

**Figure 4:** Graphical representation of the mean values in seconds (s) obtained performing Withdrawal Reflex Latency (WRL) test to evaluate the nociceptive function. This test has been performed pre-operatively (week-0), and every week during 8 weeks and then at week-10 and week-12. Results are presented as mean and standard error of the mean (SEM). N corresponds to the number of rats within the experimental group (N = 7).

**Figure 5:** Graphical representation of the mean values of Static Sciatic Function (SSI) measured pre-operatively (week-0), and every week during 8 weeks and then at week-10 and week-12. An index score of 0 is considered normal and an index of 100 indicates total impairment. The measurements of the toe spread (TS), and the intermediary toe spread (ITS), were taken from the experimental (E) and normal (N) sides. Results are presented as mean and standard error of the mean (SEM). N corresponds to the number of rats within the experimental group (N = 7).

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**Figure 7:** Kinematic plots in the sagittal plane for the ankle angle (º) as it moves through the stance phase, during the transection injury study. The mean of each group is plotted.

**Figure 8:** Histological appearance of regenerated nerve fiber in the different groups of axonotmesis: *PVA* **(A)**; *PVA-PPY* **(B)**; *PVA-CNTs* **(C)**. Magnification: 1000x; scale bar = 20 µm**.** Stereological quantitative assessment - total number of myelinated fibers, axon diameter, myelin thickness, ratio axon diameter and fiber diameter (d/D, g-ratio) of regenerated sciatic nerve fibers at week-12 after surgery. Values are presented as mean ± SEM.* $P \leq 0.05$; ** $P \leq 0.01$ **(D)**.

**Figure 9:** Graphical representation of the mean of area (**A**) and "minimal Feret's diameter" (**B**) of *Pre Crush*, regenerated TA muscle fibers at week-12 after axonotmesis (untreated or *Control*) and axonotmesis with the sciatic nerve enwrapped in different PVA membranes (*PVA*, *PVA-PPy* and *PVA-CNTs*). Values are presented as mean ± SEM.

**Figure 10:** Histological analysis of spleen, liver, kidney, lung and lymph nodes collected at week-12, from animals of the experimental group *PVA-CNTs*. Magnification: 100x; scale bar = 10 µm. **HE:** hematoxylin-eosin; **VK:** Van-Kossa; **MF:** Masson Fontana staining.

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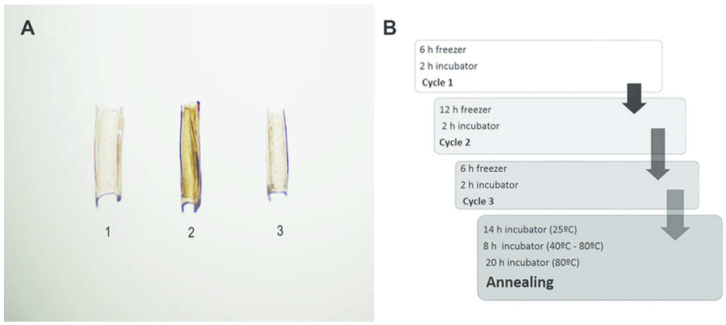


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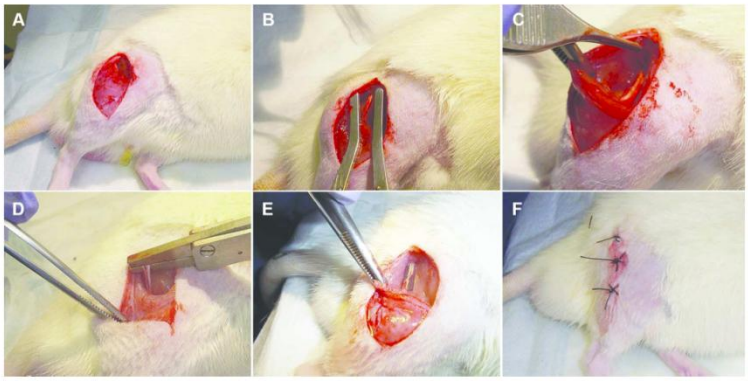


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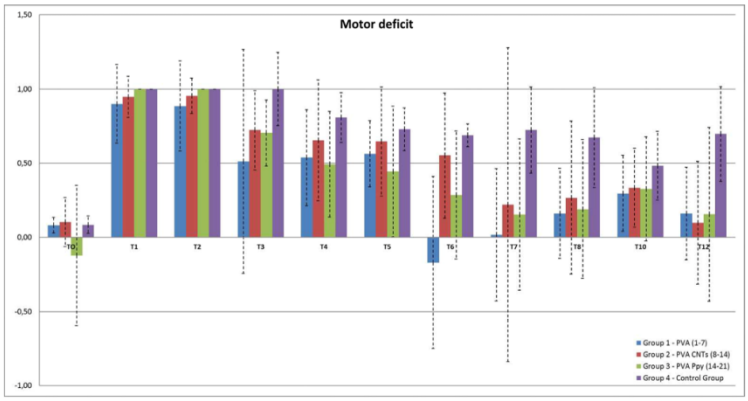


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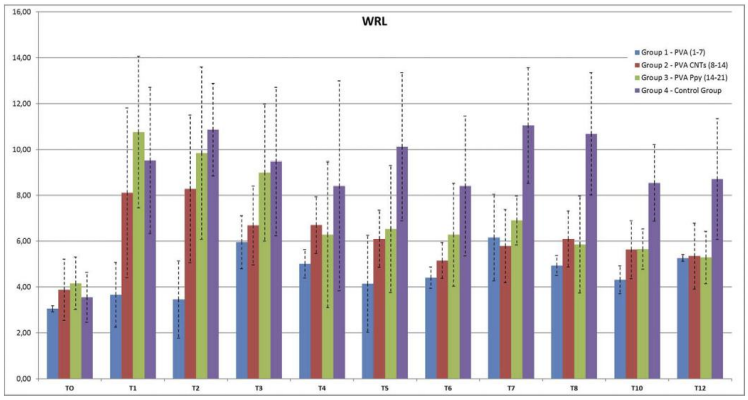


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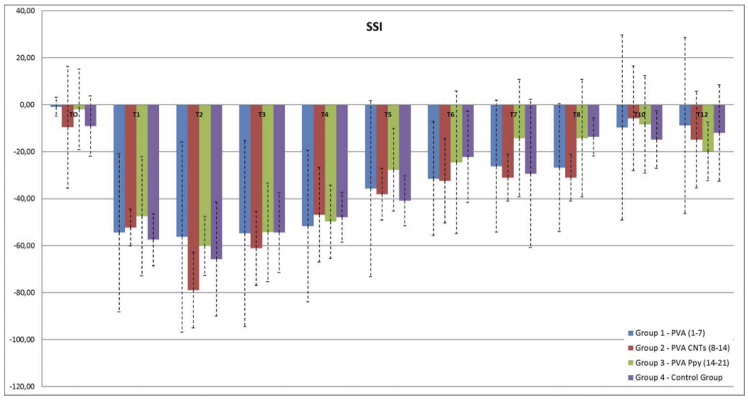


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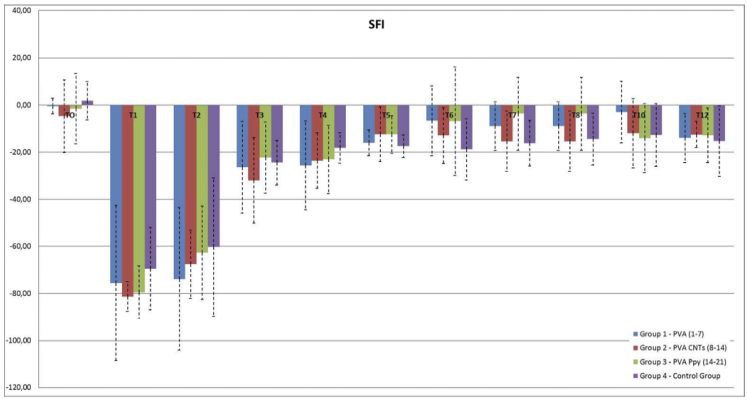


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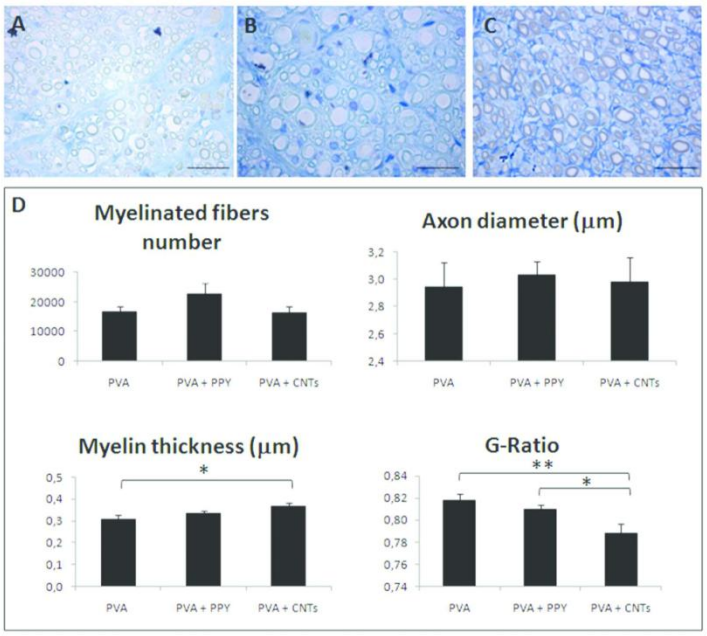


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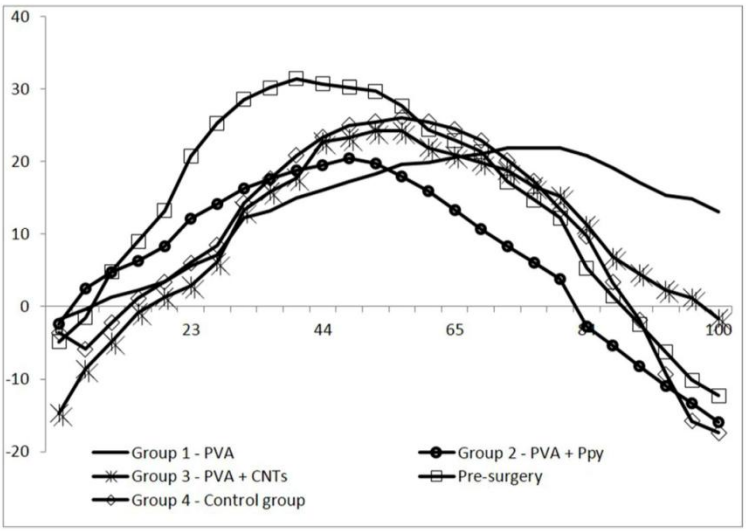


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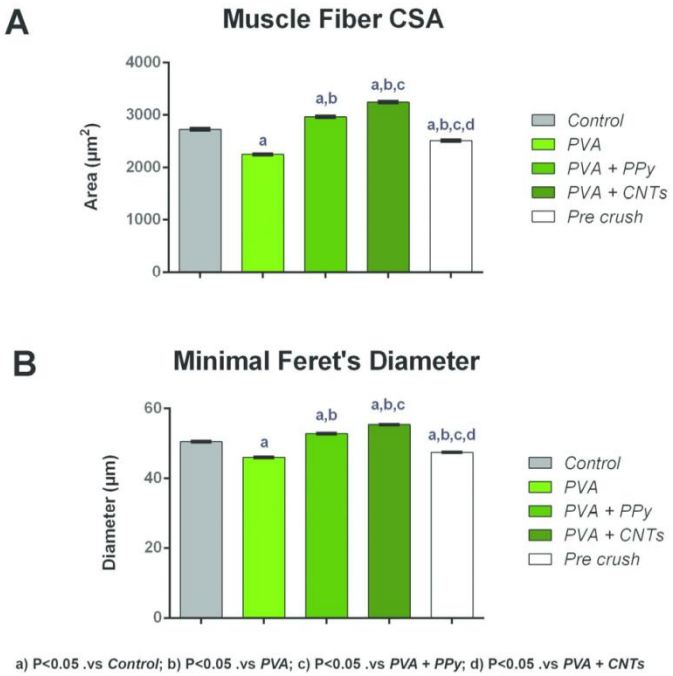
180x126mm (300 x 300 DPI)

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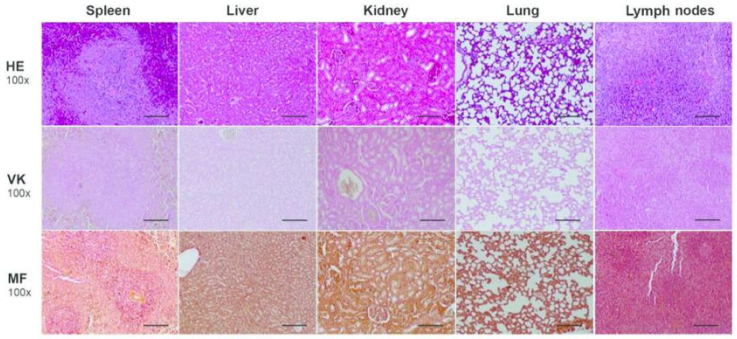


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180x83mm (300 x 300 DPI)

| Sample | Conductivity (S/m)*10^6 |
|---|---|
| PVA 15% | 1,46+/-0,49 |
| PVA 15% + PPy 0,025% | 579+/-0,607 |
| PVA 15% + CNTs 0,05% -COOH | 1250+/-0,707 |

**Table 1** - Measurements of the *in vitro* electrical conductivity of the 3 membranes: *PVA*, *PVA-PPy* and *PVA-CNTs*.

| Temporal events | Group | Result |
|---|---|---|
| IC | *Group 1* - PVA | -0,10±12,86 |
| | *Group 2* - PVA+Ppy | 4,25±7,45 |
| | *Group 3* - PVA+CNTs | 6,12±5,46 |
| | Pre crush | 5,82±1,33 |
| | *Group 4* - Control Group | 4,68±2,25 |
| OT | *Group 1* - PVA | 9,84±14,67 |
| | *Group 2* - PVA+Ppy | 14,84±5,08 |
| | *Group 3* - PVA+CNTs | 11,11±4,64 |
| | Pre crush | 8,92±4,93 |
| | *Group 4* - Control Group | 7,40±3,15 |
| HR | *Group 1* - PVA | 22,88±4,83 |
| | *Group 2* - PVA+Ppy | 22,57±17,58 |
| | *Group 3* - PVA+CNTs | 16,97±8,45 |
| | Pre crush | 16,39±5,86 |
| | *Group 4* - Control Group | 11,92±5,61 |
| TO | *Group 1* - PVA | 11,43±12,79 |
| | *Group 2* - PVA+Ppy | -15,93±3,09 |
| | *Group 3* - PVA+CNTs | -1,62±8,04 |
| | Pre crush | -3,20±11,68 |
| | *Group 4* - Control Group | -11,83±12,84 |

**Table 5:** Ankle kinematics – values were obtained performing video analysis at week-12 of the healing period. Results are presented as mean and standard deviation (SD) (N = 7). For each step cycle the following time points were identified: initial contact (IC), Opposite Toe off (OT), and Heel Rise (HR) and Toe-off (TO).

### *2.2.2.* Lesão de neurotmese - *Evaluation of biodegradable electric conductive tube-guides and mesenchymal stem cells.*

As lesões de neurotmese são aquelas em que a medicina regenerativa pode fazer a diferença. Neste trabalho experimental estudou-se a regeneração do nervo periférico recorrendo a tubos-guia de PVA, PVA com polipirrol, PVA com CNTs e PVA com CNTs em associação com aplicação local de MSCs isoladas da geleia de Wharton do tecido do cordão umbilical. Os resultados deste estudo encontram-se descritos no artigo científico:

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## Evaluation of biodegradable electric conductive tube-guides and mesenchymal stem cells

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ORIGINAL ARTICLE

Basic Study

# Evaluation of biodegradable electric conductive tube-guides and mesenchymal stem cells

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Institutional review board statement: The article describes a basic research study involving animal subjects and was reviewed and was approved by the Instituto de Ciências Biomédicas Abel Salazar (ICBAS) - University of Porto (UP) and CECA-ICETA scientific boards. All procedures involving the experimental animals were performed with the approval of the Veterinary Authorities of Portugal in accordance with the European Communities Council Directive of November 1986 (86/609/EEC), and the NIH guidelines for the care and use of laboratory animals have been observed.

Institutional animal care and use committee statement: The article describes a basic research study involving animal subjects and was approved by the Veterinary Authorities of Portugal in accordance with the European Communities Council Directive of November 1986 (86/609/EEC), and the NIH guidelines for the care and use of laboratory animals have been observed. Also all the authors involved in the *in vivo* tasks have a degree in Veterinary Medicine and are accredited by the Veterinary Authorities of Portugal (Direcção Geral de Alimentação e Veterinária - DGAV) and by Felasa - Category C for working with laboratory animals. Humane end points were always followed in accordance to the OECD Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation (2000).

Adequate measures were taken to minimize pain and discomfort taking into account human endpoints for animal suffering and distress. Animals were housed for two weeks before entering the experiment.

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Data sharing statement: This work is original in that it has not been published before or submitted for publication elsewhere, and will not be submitted elsewhere before a decision has been taken as to its acceptability in this Journal where you are Editor. Each author meets the criteria for authorship and assumes the corresponding responsibility. Technical appendix, statistical code, and dataset available from the corresponding author at Dryad repository, who will provide a permanent, citable and open-access home for the dataset is provided.



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## Abstract

**AIM:** To study the therapeutic effect of three tube-guides with electrical conductivity associated to mesenchymal stem cells (MSCs) on neuro-muscular regeneration after neurotmesis.

**METHODS:** Rats with 10-mm gap nerve injury were tested using polyvinyl alcohol (PVA), PVA-carbon nanotubes (CNTs) and MSCs, and PVA-polypyrrole (PPy). The regenerated nerves and tibialis anterior muscles were processed for stereological studies after 20 wk. The functional recovery was assessed serially for gait biomechanical analysis, by extensor postural thrust, sciatic functional index and static sciatic functional index (SSI), and by withdrawal reflex latency (WRL). *In vitro* studies included cytocompatibility, flow cytometry, reverse transcriptase polymerase chain reaction and karyotype analysis of the MSCs. Histopathology of lung, liver, kidneys, and regional lymph nodes ensured the biomaterials biocompatibility.

**RESULTS:** SSI remained negative throughout and independently from treatment. Differences between treted groups in the severity of changes in WRL existed, showing a faster regeneration for PVA-CNTs-MSCs ($P < 0.05$). At toe-off, less acute ankle joint angles were seen for PVA-CNTs-MSCs group ($P = 0.051$) suggesting improved ankle muscles function during the push off phase of the gait cycle. In PVA-PPy and PVA-CNTs groups, there was a 25% and 42% increase of average fiber area and a 13% and 21% increase of the "minimal Feret's diameter" respectively. Stereological analysis disclosed a significantly ($P < 0.05$) increased myelin thickness (M), ratio myelin thickness/axon diameter (M/d) and ratio axon diameter/fiber diameter (d/D; g-ratio) in PVA-CNT-MSCs group ($P < 0.05$).

**CONCLUSION:** Results revealed that treatment with MSCs and PVA-CNTs tube-guides induced better nerve fiber regeneration. Functional and kinematics analysis revealed positive synergistic effects brought by MSCs and PVA-CNTs. The PVA-CNTs and PVA-PPy are promising scaffolds with electric conductive properties, bio- and cytocompatible that might prevent the secondary neurogenic muscular atrophy by improving the reestablishment of the neuro-muscular junction.

**Key words:** Nerve regeneration; Neurotmesis; Tube-guides; Mesenchymal stem cells; Carbon nanotubes; Functional assessment; Gait biomechanical analysis; Histomorfometry; Polyvinyl alcohol



**Core tip:** The rat sciatic injury neurotmesis injury model is an appropriate model to evaluate the nerve regeneration when electric conductive tube-guides of polyvinyl alcohol (PVA)-carbon nanotubes (CNTs) and PVA-polypyrrole (PPy) associated to mesenchymal stem cells (MSCs) are used as scaffolds. The results obtained revealed that treatment with MSCs associated to PVA-CNTs tube-guides induced an increased number of regenerated fibers and thickening of the myelin sheet. Functional and kinematics analysis revealed positive synergistic effects brought by MSCs and PVA-CNTs. The PVA-CNTs and PVA-PPy are promising scaffolds with electric conductive properties, bio- and cytocompatible that might prevent the secondary neurogenic muscular atrophy by improving the reestablishment of the neuro-muscular junction.

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## INTRODUCTION

Functional recovery is rarely satisfactory in patients where peripheral nerve repair is needed, remaining a challenging task in neurosurgery[1,2]. Direct repair is the procedure of choice but only when a tension-free end-to-end suture is possible. However, for patients with loss of nerve tissue, resulting in a gap it is necessary to reconstruct the injured nerve using an autograft or a graft from a compatible donor[1,3]. One of the disadvantages of grafting is the necessity of a second surgery for collecting the nerve sample and respective donor site morbidity. In addition, non-matching donor and recipient nerve diameters often occur, which might be the reason for an incomplete functional recovery[4]. Entubulation offers advantages compared to graft implantation, including the potential to manipulate and to improve the regeneration environment within the tube-guide by adding to the lumen, growth factors and/or cellular systems[5]. Consequently, guidance of regenerating axons is improved by a mechanical effect but also by cellular growth factors, a chemical[6] and electrical cues[7]. Natural and synthetic biomaterials have been used as tube-guides, being the later sub-divided into two major groups: biodegradable and non-biodegradable[8,9]. The development of tube-guides is a consequence of the limitations inherent in the use of grafts, in terms of length, diameter and type of fiber, preventing damage to the sampling local, as it was previously referred[10]. For a correct nerve regeneration some important physical properties of tube-guides have been listed and referred by several authors like: the tube-guides should be made of biodegradable biomaterials with appropriate porous dimensions; the ability to deliver growth factors and drugs and allow the incorporation of cellular systems; an internal geometry to support an organized cell migration or intraluminal structures similar to nerve fascicles; and with some electrical activity in order to promote axon regeneration[11]. The use of electric conductive biomaterials to achieve nerve regeneration is a promising research area[12]. The goal of this work was to evaluate the therapeutic effect (by morphological and functional analysis) of three previously developed tube-guides with electrical conductivity for nerve regeneration associated to mesenchymal stem cells (MSCs) isolated from the Wharton's jelly of umbilical cord (UC) on neuro-muscular regeneration after neurotmesis injury using the rat sciatic nerve model. The three tube-guides used *in vivo* where those made of polyvinyl alcohol (PVA), PVA loaded with MWCNTs [functionalized carbon nanotubes (CNTs), PVA-CNTs], and PVA loaded with polypyrrole (PVA-PPy). Such composites with electric conductivity can be used to host cell therapies, and beneficial regenerative electrical stimulation can be applied directly to the cells through the composite[13]. PVA is a polymer used as a biomaterial due to its biocompatibility, non-toxic, non-carcinogenic, swelling properties, and bio-adhesive characteristics. PVA can also be used as host material in order to increase the solubility as well as the mechanical strength of conductive materials, and it is approved by Food and Drug Administration[14,15]. PPy and CNTs are two of the most studied conductive polymers for tissue engineering, especially for nerve tissue engineering[16]. The authors previously to *in vivo* application, focused on tube-guides design and *in vitro* characterization. The PVA-CNTs and PVA-PPy tube-guides presented conductivity advantages, important for nerve regeneration. The developed electrical conductive nerve tube-guides was achieved by loading PVA with 0.05% of PPy or COOH-functionalized CNTs. The inclusion of CNTs and PPy brought a significant increase of electrical conductivity of the simple PVA tube-guide. The PVA-CNTs tube-guides showed the rougher topography. The DSC and X-ray diffraction (XRD) studies revealed that all materials have similar low crystallinity. The wettability studies indicated a hydrophilic behaviour of all nerve tube-guides, being the PVA-PPy slightly more hydrophobic. In terms of surface charge, the zeta potential measurements revealed that both COOH-functionalized CNTs and PPy loads turned the surface charge slightly more positive. Regarding the elastic behaviour, the COOH-functionalized CNTs load caused a slight decrease of the rigidity of PVA. PVA-CNTs and PVA-PPy might present a regenerative potential and were tested in the rat sciatic nerve neurotmesis injury model[17]. Also, since the PVA-CNTs presented the rougher topography, which is important for the association and viability of the associated cellular systems[9], where also tested associated to the MSCs-based therapy[3,18].

Considering the peripheral nerve system (PNS), MSCs are promising cell-based therapies to be applied alone or associated to scaffolds. MSCs have a high plasticity, proliferative and differentiation capacity, and also have the advantage of presenting immuno-suppressive properties[3,19,20]. For these reasons, MSCs have been used in experimental trials as cell-based therapies, including pathologies of PNS and central nervous system (CNS). Furthermore, nowadays the characterization of MSCs is well defined by recommendations and standards stated by the international society for cellular therapy (ISCT)[19]. The MSCs therapeutic effect resides on their capacity to replace original cells of damaged tissues, and also by the physiologic secretion of growth factors and cytokines that modify the microenvironment inducing the activity of endogenous progenitor cells within the injured tissue, and by modulating the inflammatory and immune responses. The inflammatory modulation includes the Wallerian degeneration, a crucial step for nerve regeneration after neurotmesis injury[3]. Therefore, the use of cellular systems is a rational approach for promoting nerve

**Figure 1 Synthetic biodegradable tubes of polyvinyl alcohol, polyvinyl alcohol loaded with COOH-functionalized multiwall carbon nanotubes MWCNTs (polyvinyl alcohol-carbon nanotubes, polyvinyl alcohol-carbon nanotubes tube-guides), and polyvinyl alcohol loaded with polypyrrole (polyvinyl alcohol-polypyrrole, polyvinyl alcohol-polypyrrole tube-guides).**

regeneration by delivering growth-promoting factors and cytokines at the nerve lesion site[9]. MSCs can be isolated from several tissues, including bone marrow (BM), umbilical cord blood (UCB) and umbilical cord tissue (UCT), dental pulp, and adipose tissue[9]. BM collection has several disadvantages, since the BM include a high percentage of adipocytes and a heterogeneous cell population, there is the possibility of morbidity associated to donor collection, as well as it is observed a decreasing number of BM-MSCs along the adult life and available for therapeutic applications. The research concerning other sources of MSCs has been intensively performed for the past years for identifying tissues that will allow MSCs isolation, and safe and controlled *ex vivo* expansion of these cells for potential allogeneic and autologous application[9]. MSCs isolated from the Wharton's jelly UCT have been tested for the clinical application in CNS and PNS, including neurodegenerative diseases[21]. It has been reported that MSCs from the UCT are still viable 4 mo after transplantation and there is no need for immunological suppression of the patients and experimental animals[22]. The reason for this advantage is the fact that these cells are negative for major histocompatibility complex (MHC), and have low expression of MHC class I [19] with potential application for MSC-based therapies in allogeneic treatments. *In vivo* studies involving these MSCs are limited but encouraging, especially what concerns PNS. Also, there are an increasing number of high quality cryopreserved cord tissue units in Cord Blood Banks worldwide. In addition, these cells represent a non-controversial source of MSCs, without neither ethical nor religious issues that are routinely harvested after birth, cryogenically stored, thawed, and can be expanded for therapeutic uses[9]. On the other hand, it was demonstrated in some studies, that grafted MSCs have a signalling role which initiates the recruitment and direction of endogenous cells by growth factors production, promoting the local regeneration and modulating the inflammatory process[23]. Nowadays it is believed that factors secreted by MSCs are primarily responsible for their therapeutic action, so it is particularly important to understand and fully characterize the MSCs secretome[18,24,25]. Recent studies demonstrated that MSCs produce and secrete multiple paracrine factors like interleukine-2 (IL-2), IL-6, IL-8, IL-12, IL-15, monocyte chemoattractant protein-1, macrophage inflammatory protein-1β, regulated on activation, normal T cell expressed and secreted, and platelet-derived growth factor-AA. The several secreted factors previously listed that are present in MSCs conditioned medium (CM) was already described by others[26] and more recently, by our research group[18]. These paracrine factors are important to promote the MSCs therapeutic effects identified by the scientific community, like immunomodulatory and chemoattractive, anti-apoptotic, anti-fibrotic, angiogenic, and anti-oxidants activities. The hypothesis that MSCs and the growth factors produced during expansion in culture (CM) is an appropriate therapeutic product for local application in severe peripheral lesions seemed to be a rational approach and was studied in the present experimental work.

## MATERIALS AND METHODS

### *Tube-guide preparation*

Synthetic biodegradable tubes of PVA (Aldrich, Mowiol 10-98), PVA loaded with COOH-functionalized multiwall carbon nanotubes MWCNTs (Nanothinx, NTX5, MWCNTs 97% - COOH) (PVA-CNTs tube-guides), and PVA loaded with PPy (Aldrich, 10-40 S/cm of conductivity) (PVA-PPy tube-guides) were prepared using a casting technique to a silicone mould. A 15% (%w/v) aqueous solution of PVA was prepared. Then the solution of PVA was mixed with 0.05% of COOH-functionalized MWCNTs and 0.05% of PPy. The tube-guides were produced by freezing/thawing process. The treatment consisted in three cycles of freezer (-30 ℃)/ incubator (25 ℃), and an annealing treatment started with a stage of 14 h on an incubator (25 ℃) followed by a ramp rate of 0.1 ℃ /min until 80 ℃, and then a stage of 20 h at 80 ℃. The tube-guides were sterilized by gama-radiation and hydrated in a sterile saline solution during 2 h before microsurgical application in the rat neurotmesis injuries (Figure 1).

### *MSCs cell culture and in vitro characterization*

**MSCs culture and expansion:** Human MSCs isolated from the Wharton jelly (WJ) of UCT from PromoCell GmbH (C-12971, lot-number: 8082606.7) were cultured and expanded in a humidified atmosphere with 5% $CO_2$ at 37 ℃ by replacing the mesenchymal stem cell medium, PromoCell (C-28010) every 48 h. At 80% confluence, normally obtained after 4 d in culture (Figure 2), MSCs were harvested with 0.25% trypsin with EDTA (GIBCO) for further expansion at an initial concentration of 1 × $10^4$ cells/$cm^2$. Immediately previously to *in vivo* application, a MSCs suspension was prepared in 1 mL syringes. Each syringe contained MSCs at a concentration

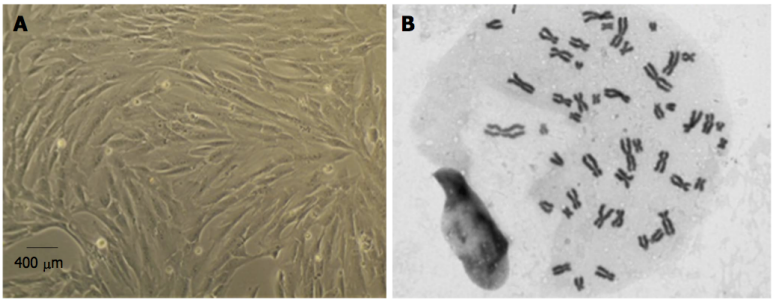


**Figure 2 Mesenchymal stem cells culture and expansion.** A: Isolated mesenchymal stem cells (MSCs) from the Wharton's jelly of umbilical cord presented a fusiform, fibroblast-like morphology in culture (magnification: 100 ×); B: Selected metaphases from MSCs in culture, showing normal number of chromossomes (46, XY) (magnification: 1000 ×).

of 1 × $10^6$/μL for posterior intra-operatively nerve injection.

The MSCs lot used in the present experimental study was characterized by flow cytometry analysis for a comprehensive panel of markers by PromoCell as previously reported[18,25]. The MSCs exhibited a mesenchymal-like shape with a flat and polygonal morphology during cell culture expansion. Their phenotype was evaluated by flow cytometry for PE anti-human cluster of differentiation-105 (CD105); APC anti-human CD73; PE anti-human CD90; PerCP/Cy5.5 anti-human CD45: FITC anti-human CD34; PerCP/Cy5.5 anti-human CD14; Pacific Blue anti-human CD19 and pacific-blue anti-human Human Leukocyte Antigen-DR (HLA-DR) (antibodies and their respective isotypes from BioLegend).

**MSCs karyotype by cytogenetic analysis:** Cytogenetic analysis was carried out before *in vivo* application between passages 4 and 5 as previously reported[18,25]. Chromosome analysis was performed by one scorer on 20 Giemsa-stained metaphases and each MSC cell was scored for chromosome number. For determination of the karyotype, a routine chromosome G-banding analysis was also carried out and no structural alterations were observed which demonstrates the chromosomal stability to the cell culture procedures and also that the *in vivo* applied MSCs are not neoplastic (Figure 2).

**Cytocompatibility evaluation of biomaterials:** Intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) by using dual wavelength spectrofluorometry as previously described[27] was measured in Fura-2-loaded MSCs cells. $[Ca^{2+}]_i$ from MSCs cultured without the presence of any biomaterial, sub-cultured over PVA discs, over PVA loaded with CNTs (PVA-CNTs) and PVA loaded with PPy (PVA-PPy) discs of 10 mm diameter are results obtained from epifluorescence technique confirming that the MSCs did not begin the apoptosis process.

**Reverse transcriptase polymerase chain reaction to confirm that MSCs are undifferentiated:** Reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative PCR (qPCR) targeting specific genes expressed by the MSCs that were *in vivo* applied was performed to certify that the MSCs used *in vivo* were not differentiated. For that, primers were designed targeting seven human genes based on the literature[18]. DNA sequences from growth associated protein-43 (GAP-43), neurofilament-H (NF-H), Nestin, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), β-actin, neuronal nuclear (*NeuN*) and glial fibrillary acidic protein (*GFAP*) genes from mice (*Mus musculus*), rat (Rattus novergicus) and human (Homo sapiens) were downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank) and aligned using the Clustal Omega bioinformatic tool from EMBL-EBI (http://www.ebi.ac.uk/Tools/msa/clustalo). MSCs culture was harvested with 0.25% trypsin EDTA solution (Gibco) and centrifuged at 2000 rpm 4 ℃ during 5 min. Cell pellets were used for total RNA extraction using an adequate extraction kit, high pure RNA Isolation kit (Roche). Briefly, cell pellets were lysed with a lysis buffer, loaded into a High Pure Filter Tube, DNA was removed with DNase I enzyme, washed twice on column, and eluted with 100 μL of Elution Buffer. RNA was quantified and its quality assessed by using a Nanodrop ND-1000 Spectrophotometer and reads from 220 nm to 350 nm, and then stored at -80 ℃ until further use. In the following step, cDNA was synthesized from the purified RNA. To fulfill that issue, the kit Ready-To-Go You-Prime First-Strand Beads (GE Healthcare) was used following the manufacturer instructions. Briefly, 1.5 μg of total RNA was used and diluted in DEPC-treated water to a 30 μL final volume in a RNase-free microcentrifuge tube; then heated at 65 ℃ for 10 min and then chilled in ice; transfer the RNA solution to the kit tube containing the first-strand reaction mix beads; add 0.2 μg of Oligo(dT) primer and DEPC-treated water to a 33 μL final volume; mix the content and incubate at 37 ℃ for 60 min. cDNA was synthesized and stored at -20 ℃ until further use. Of referring that, due to the use of the Oligo(dT) primer, the synthesized cDNA corresponds to the mRNA present in the sample at the time of collection. cDNA synthesized from undifferentiated MSCs was used to

check the expression of seven genes, two housekeeping genes (β-actin and *GAPDH*) and five specific of neuronal cells (GFAP, NeuN, Nestin, NF-H and GAP-43). Primers were designed in house and then synthesized in an external laboratory (MWG Operon, Germany). The primers were rehydrated in DNase/RNase free water in a concentration of 100 pmol/μL. qPCR was performed in a iCycler® iQ5TM (BioRad) apparatus using the iQTM SYBR® Green Supermix (BioRad). Each pair of primers targeting a gene was used to analyze its expression in the MSCs cDNA, in triplicate, along with a negative control. The plates containing the mix targeting the seven genes for both types of cells were submitted to the following cycles of temperatures: 95 ℃ during 4 min, 35 cycles comprising 95 ℃ during 20 s, 55 ℃ during 20 s and 72 ℃ during 20 s ending with Real-Time acquisition, and final extension of 75 ℃ for 7 min. After cycling temperatures, the number of cycle threshold for each well was recorded. The plate containing the amplified genes or qPCR products was kept in ice and observed in a 2% agarose gel to check and reinforce the identity of the amplicons. Briefly, 2 g of NuSieve® 3:1 Agarose (Lonza) were mixed with 100 mL Tris-Acetate-EDTA buffer, melted, mixed with ethidium bromide in a final concentration of 0.2 μg/mL, and loaded in a horizontal electrophoresis apparatus. After solidification, 15 μL of the qPCR products were loaded in the agarose wells, and submitted to a 120 V potential difference during 40 min to separate the amplicons. Gel was then observed under UV light and pictures recorded using the GelDoc® 2000 (BioRad) and Quantity One® software (BioRad). In the MSCs, the molecular analysis showed a very small amplification of *GFAP* gene, absence of amplification of the *NF-H* and *GAP-43* genes, and reasonable amplification of *NeuN*, β-actin, *GAPDH* and Nestin genes. Amplification of a given gene is correlated with its expression seeing that the template DNA is the one generated from mRNA. The RT-PCR results confirmed that the MSCs used *in vivo* were not differentiated into neuro-glial cells.

### *Surgical procedure for the rat sciatic nerve model for neurotmesis*

The article describes a basic research study involving animal subjects and was approved by the Veterinary Authorities of Portugal [Direcção Geral de Alimentação e Veterinária (DGAV)] in accordance with the European Communities Council Directive of November 1986 (86/609/EEC), and the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals have been observed. Also all the authors involved in the *in vivo* tasks have a degree in Veterinary Medicine and are accredited by the Veterinary Authorities of Portugal (DGAV) and by Felasa - Category C for working with laboratory animals.

The OECD Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation (2000) were always followed by the authors by taking adequate measures to minimize pain and discomfort.

Polycarbonate cages type 3 were used for housing under standard laboratory conditions adult male Sasco Sprague Dawley rats with 300 g (Charles River Laboratories, Barcelona, Spain) always in a temperature and humidity controlled room with 12-12 h light/dark cycles. The rats were fed with standard chow and water *ad libitum* until the day of surgery. For surgery, the rats were anesthetized with ketamine 9 mg/100 g and xylazine 1.25 mg/100 g (body weight), by intraperitoneal administration. In lateral recumbence, the right sciatic nerve was exposed unilaterally and a transection injury was performed above the terminal nerve ramification using a straight microsurgical scissors, for creating a neurotmesis injury with 10 mm gap. Six experimental groups were studied: in groups 1, 2 and 3 after neurotmesis the proximal and distal nerve stumps were inserted 3 mm into PVA (group 1: PVA), PVA loaded with 0.05% (%w/v) of COOH-functionalized MWCNTs (group 2: PVA-CNTs), and PVA loaded with PPy 0.05% (%w/v) tube-guides (group 3: PVA-PPY), respectively, and tube-guides were sutured with two epineural sutures using 7/0 polypropylene monofilament, maintaining a nerve gap of 10 mm; in group 4 after neurotmesis, an autologous 180° inverted graft was sutured between both nerve stumps (group 4: Graft); in group 5 after neurotmesis, immediate cooptation with 7/0 monofilament polypropylene suture (group 5: End-to-End); in group 6 after neurotmesis the proximal and distal nerve stumps were inserted 3 mm in a PVA loaded with 0.05% (%w/v) of COOH-functionalized MWCNTs tube-guide kept in place with 2 epineural sutures using 7/0 monofilament polypropylene suture and the interior of the tube-guide was filled with 100 μm of MSCs at a concentration of $1 \times 10^6$ cells/μL in culture medium, also 100 μm of MSCs at a concentration of $1 \times 10^6$ cells/μL in culture medium were infiltrated in both nerve stumps inserted in the tube-guide (group 6: PVA-CNTs-MSCs) (Figure 3).

### *Functional assessment*

After injury and sciatic nerve microsurgery reconstruction using the developed scaffolds in standardized neurotmesis injuries, a follow-up consisting in functional parameters measurements are important to evaluate the regeneration process and recovery. The animals were tested in an appropriate environment to minimize stress and handled by a single operator already trained for that purpose. Animals have been tested before the surgery at week 0, and after the surgery, at week 1, at week 2 and then every two weeks until the end of follow-up time of 20 wk.

**Evaluation of motor performance and nociceptive function:** Motor performance was evaluated by measuring extensor postural thrust (EPT) and nociceptive function using the withdrawal reflex latency (WRL). The EPT included in the neurological recovery evaluation

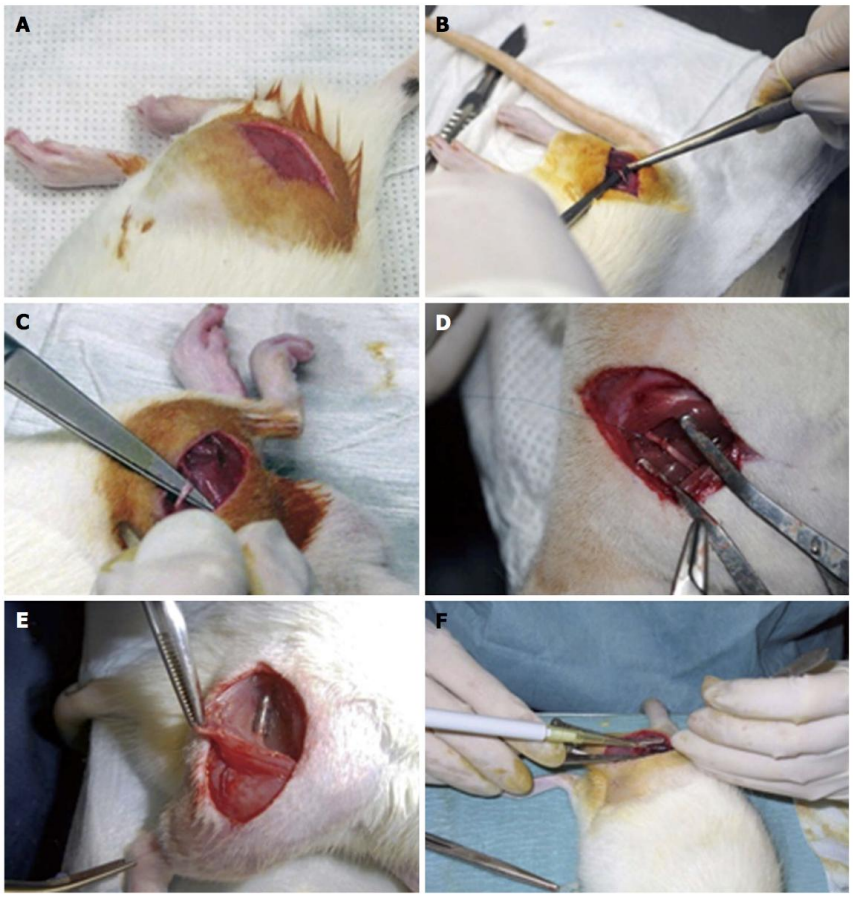


**Figure 3 Surgery of the rat sciatic nerve neurotmesis injury model.** Under deep anesthesia the right sciatic nerve was exposed unilaterally through a skin incision extending from the greater trochanter to the mid-half distally followed by a muscle splitting incision (A). After nerve mobilization (B), a transection injury was performed (neurotmesis) using a straight microsurgical scissors (C). In group 5 (End-to-End) after neurotmesis, immediate cooptation with 7/0 monofilament polypropylene suture was performed (D). Implantation of the tube-guide in the 10 mm gap (E). Local application of the mesenchymal stem cells (MSCs), the MSCs suspension filled the polyvinyl alcohol (PVA)-carbon nanotubes (CNTs) tube-guide lumen (group 6: PVA-CNTs-MSCs) (F).

of the rat after sciatic nerve injury was first described by[28]. EPT is induced by lowering the affected hind-limb towards the platform of a digital balance supporting the animal by the thorax. During the test, the rat extends the hind-limb and the distal metatarsus and digits contact with digital platform balance. The force in grams (g) applied is recorded individually and the results for both hind-limbs (affected and un-affected hind-limb) are taken for equation 1 calculation. The normal (unaffected limb) EPT (NEPT) and experimental EPT (EEPT) values considered for equation (1) and the percentage of functional deficit is calculated[9,28-31].

$$\% \text{ Motor deficit} = [(\text{NEPT} - \text{EEPT})/\text{NEPT}] \times 100 \quad (1)$$

To assess the nociceptive WRL, the hotplate test described by Masters *et al*[32] was used with some modifications and previously described by the authors[3,9,17,25,31,33]. Also, it is considered by the scientific bibliography that rats without sciatic nerve injury withdraw their paws from the hotplate within 4.3 s or less[9,17,31,33]. If there was no paw withdrawal after 12 s, the animal was assigned the maximal WRL of 12 s and the heat stimulus was removed to prevent tissue damage[34-36].

For sciatic functional index (SFI), a confined walkway measuring 42 cm long and 8.2 cm wide with a dark shelter at the end (own fabrication) that the rats cross was used. The footprints from the experimental (E) and normal (N) sides are measured: (1) distance from the heel to the third toe, the print length; (2) distance from the first to the fifth toe, the toe spread (TS); and (3) distance from the second to the fourth toe,

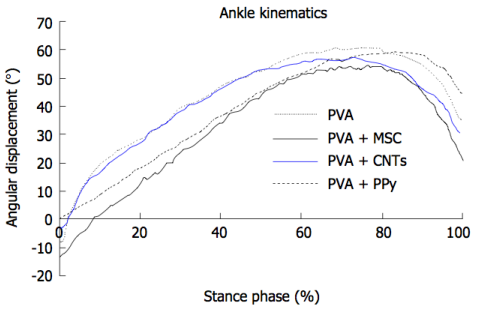


**Figure 4 Kinematic plots in the sagittal plane for the ankle angle (°) as it moves through the stance phase, during the transection injury study.** The mean of each group is plotted. PVA: Polyvinyl alcohol; CNTs: Carbon nanotubes; PPy: Polypyrrole; MSCs: Mesenchymal stem cells.

the intermediary toe spread (ITS). In the static sciatic functional index (SSI) is a simpler test where only the parameters TS and ITS are the measurements included in equation (6). Prints for measurements are chosen at the time of walking based on precise, clear and completeness of footprints[9,36,37]. The mean distances of 3 measurements are used to calculate the following factors (dynamic and static):

$$TSF = (ETS - NTS)/NTS \quad (2)$$

$$ITSF = (EITS - NITS)/NITS \quad (3)$$

$$PLF = (EPL - NPL)/NPL \quad (4)$$

TSF: Toe spread factor; ITSF: Intermediate toe spread factor; PLF: Print length factor; ETS: Experimental toe spread; NTS: Normal toe spread; NITS: Normal intermediary toe spread; EITS: Experimental Intermediary toe spread; NPL: Normal print length; EPL: Experimental print length.

SFI is calculated as described by Bain *et al*[38] according to the following equation:

$$SFI = -38.3 \times (EPL - NPL)/NPL + 109.5 \times (ETS - NTS)/NTS + 13.3 \times (EITS - NITS)/NITS - 8.8 = (-38.3 \times PLF) + (109.5 \times TSF) + (13.3 \times ITSF) - 8.8 \quad (5)$$

Considering the SFI measurements, it was not possible to obtain values during the 20 wk follow-up after the surgery procedure, due to autotomy of the fingers. As a matter of fact, the rat sciatic nerve model for neurotmesis has this disadvantage; the autotomy observed in several experimental animals even when a repellant substance is applied routinely[9]. SSI was calculated as described by[39] according to the following equation (equation 6):

$$SSI = 108.4 \times TSF + 31.85 \times ITF - 5.49 \quad (6)$$

For SFI and SSI, when no footprints are measurable, the index score of -100 is given considering total impairment, on the other hand, a score of 0 means that there is no alteration and the footprint is normal.

**Kinematic analysis**: Ankle kinematics was carried out at the end of the 20-wk follow-up time in the following experimental groups: PVA, PVA-CNTs, PVA-PPy, Graft, End-to-End, and PVA-CNTs-MSCs. A Perspex track with length, width (adjustable) and height of 120, 12, and 15 cm, respectively was used to record the rat locomotion in a straight line. The animals' gait was video recorded at a rate of 300 Hz images per second (Casio Exilim PRO EX-F1, Japan) by a camera with a visualization field of 14 cm wide, that was positioned at the track half-length, and 1 m distant from the track, where gait velocity was steady. The software APAS® (Ariel Performance Analysis System, Ariel Dynamics, San Diego, United States) was used for data analysis. The authors used a two-segment model of the ankle joint, adopted from the model firstly developed by[40] (2D biomechanical analyses in sagittal plan), and the rat ankle angle was determined using the scalar product between a vector representing the foot and a vector representing the lower leg. Dorsiflexion and plantarflexion is considered for positive and negative values of position of the ankle joint (θ°). Initial contact (IC), opposite toe off (OT), and heel rise (HR) and toe-off (TO)[33,40] were the time points analyzed for each step cycle, the values were time normalized for 100% of step cycle. The normalized temporal parameters were averaged over all recorded trials (Figure 4). Since the animal's normal walking velocity is 20-60 cm/sA[33,40], stance phases lasting between 150 and 400 ms were considered for analysis.

**Morphological analysis and histopathology**: For histomorphometric analysis, nerve samples obtained from the 10-mm-long sciatic nerve segments distal to the neurotmesis site and from un-operated controls[41-44]. The histological nerve preparation followed the previously reported protocol[3,9,17,25,31,33,35] and a systematic random sampling and D-disector were adopted[41-44].

At the end of the healing period tested (week 20), tibialis anterior (TA) muscles of all experimental (PVA, PVA-CNTs, PVA-PPy, Graft, End-to-End, and PVA-CNTs-MSCs) and Control (no lesion) groups were collected, and the tissue samples were fixed in 10% buffered formalin, routinely processed, dehydrated and embedded in paraffin wax. Consecutive 3 μm transverse sections from the mid-belly of each muscle were cut, stained with haematoxylin and eosin (HE) and used for morphometry evaluation and determination of the degree of atrophy. For the morphometric analysis, an unbiased sampling procedure was applied and the following measures were calculated: area, perimeter, "Feret's angle" and "minimal Feret's diameter" (which is the minimum distance of parallel tangents at opposing borders of the muscle fiber). These parameters were evaluated from the cross sections using the ImageJ© software (NIH) which allowed to apply this set of individual fiber measurements. A

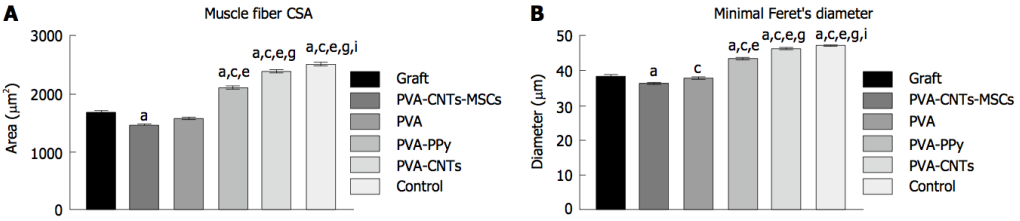


**Figure 5 Graphical representation of the mean of area (A) and "minimal Feret's diameter" (B) of control, regenerated tibialis anterior muscle fibers at week-20 after neurotmesis (Graft) and neurotmesis with the proximal and distal nerve stumps sutured to a polyvinyl alcohol (polyvinyl alcohol) tube and polyvinyl alcohol coated tubes (polyvinyl alcohol, polyvinyl alcohol polypyrrole and polyvinyl alcohol carbon nanotubes).** Values are presented as mean ± SEM. $^{a}P < 0.05$ *vs* Graft; $^{c}P < 0.05$ *vs* Graft PVA CNTs MSCs; $^{e}P < 0.05$ *vs* Graft PVA; $^{g}P < 0.05$ *vs* Graft PVA PPy; $^{i}P < 0.05$ *vs* Graft PVA CNTs. PVA: Polyvinyl alcohol; CNTs: Carbon nanotubes; PPy: Polypyrrole; MSCs: Mesenchymal stem cells.

minimum of 1000 skeletal muscle fibers was measured from each group. This assessment was performed by 2 independent operators. Each one of the operators measured, blindly and randomly, an average of 50 fibers in each section. Images were acquired using a Nikon® microscope connected to a Nikon® digital camera DXM1200, at low magnification (100 ×) under the same conditions that were used to acquire a reference ruler.

At the end of the study, all animals were subjected to a complete necropsy examination in order to evaluate the presence of possible internal anomalies and/or injuries. Lung, heart, kidneys, liver and spleen were collected and weighed and then submitted to histological analysis to check whether there were related microscopic changes such as inflammation, degeneration, or accumulation of biological material. The organs were fixed in 10% buffered formalin and processed for routine histology with HE stain. Microscopically, massive carbon deposits generally appear as well-recognized anthracotic black pigment, especially in the lung. A recent report described that some types of carbon nanotubes may appear as small punctuated accumulations inside Kupfer cells (liver) and in the intermediate zone of the spleen, when intravenously administered[45]. Special stains, such as Von Kossa (method that demonstrates phosphates and carbonates) and Masson-Fontana (method used for distinguish carbon deposits from melanin) were also performed in consecutive sections.

### *Statistical analysis*

The statistical review of the study was performed by a biomedical statistician [PAS Armada-da-Silva, CIPER-Faculdade de Motricidade Humana (FMH): Centro Interdisciplinar de Estudo de Performance Humana, FMH, Universidade de Lisboa, Estrada da Costa, 1499-002, Cruz Quebrada - Dafundo, Portugal].

A mixed model repeated measures ANOVA was used to test for differences across time and sciatic nerve treatment. Sphericity was assessed by Mauchly's test and Greenhouse-Geisser degrees of freedom correction was used in cases sphericity could not be assumed or when corrected *P*-values were below the accepted level of significance ($P < 0.05$). Tukey's HSD test was used for pairwise comparisons. All data is presented as mean and standard deviation, unless otherwise stated. These statistical tests were carried out with IBM SPSS Statistics version 19. For stereology, statistical comparisons of quantitative data were subjected to one-way ANOVA test, followed by pairwise comparisons using Tukey's HSD test. Statistical significance was established as $P < 0.05$. Stereological data was analyzed using the software using the SPSS version 19.0 (SPSS, Chicago, IL). For muscle morphometry, statistical analysis was performed using the SPSS version 19.0 (SPSS, Chicago, IL). Results are presented as mean ± scanning electron microscope (SEM) in Figures 5 and 6. Multiple comparisons between groups were performed by one-way ANOVA supplemented with Tukey's HSD post hoc test. Differences were considered statistically significant at $P < 0.05$.

## RESULTS

### *Development and characterization of MSCs isolated from UC WJ*

MSCs are defined by the ISCT which includes the following characteristics: (1) their capacity to adhere to plastic surfaces during cell culture; (2) expression of the specific surface markers (positive for CD73, CD90, and CD105, and no expression of CD14, CD19, CD34, CD45 and HLA-DR); and (3) are able to undergo tri-lineage differentiation into adipocytes, chondrocytes and osteoblasts[19]. MSCs isolated from the UCT were expanded to P5-P6 where the culture appeared homogeneous and cells presented their typical fusiform, fibroblast-like, morphology (Figure 1). Flow cytometry analysis performed previously to *in vivo* application to expanded MSCs showed that over 95% of the cells in the population were consistently positive for the cell surface markers CD44, CD73, CD90 and CD105 and less than 2% positive for CD14, CD19, CD31, CD34, CD45 and HLA-DR. Also, the phenotype of MSCs was assessed by PromoCell by flow cytometry analysis for an additional comprehensive panel of markers: PECAM (CD31), HCAM (CD44), CD45, and Endoglin (CD105). Overall, these results are the ones expected for MSC-type stem cells according to ISCT[19].

Giemsa-stained cells of MSCs at P5 were analyzed

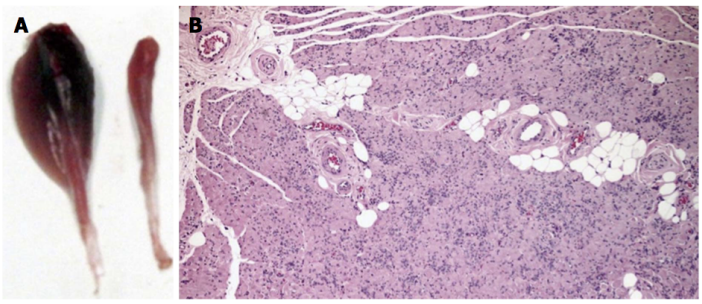


**Figure 6 Macroscopic appearance of control tibialis anterior muscle and histological haematoxylin and eosin staining of a transverse section of tibialis anterior muscle.** A: Macroscopic appearance of control tibialis anterior (TA) muscle (left) and 20 wk following neurotmesis and surgical treatment with PVA CNTs MSCs (right); B: Histological haematoxylin and eosin staining of a transverse section of TA muscle from PVA CNTs MSCs group. Magnification 40 ×. PVA: Polyvinyl alcohol; CNTs: Carbon nanotubes; MSCs: Mesenchymal stem cells.

for cytogenetic characterization. The karyotype of MSCs was determined previously to *in vivo* application to ensure that no neoplastic cells were used proving the cell therapy safety. The karyotype analysis demonstrated that no structural alterations were found, as well as chromosomal stability in terms of number and structure of the somatic and sexual chromosomes, to the cell culture procedures. The transplanted MSCs also presented normal morphology and flow cytometry markers for MSCs according to ISCT[19].

Results obtained from epifluorescence technique confirmed that MSCs did not begin the apoptosis process, showing that the *in vivo* applied MSCs were viable even in the presence of the tube-guides biomaterials. The $[Ca^{2+}]_i$ measured was 47.9 ± 4.5 ($n$ = 25), 46.2 ± 3.5 ($n$ = 25) and 48.1 ± 3.9 ($n$ = 25) for MSCs cultured in the presence of PVA, PVA-CNTs, and PVA-PPy discs after 7 d of culture, respectively. The MSCs cultured and expanded in the presence of the three tested biomaterials reached confluence and exhibited a normal star-like shape with a flat morphology in culture. According to these results, it is reasonable to conclude that the three biomaterials (PVA, PVA-CNTs, and PVA-PPy) are viable substrate for MSCs culture and survival and may be used in the pre-clinical trials.

The MSCs were harvested and its RNA purified and converted to cDNA using adequate procedures. Primers targeting markers, two housekeeping genes (β-actin and *GAPDH*) and five specific of neuronal cells (GFAP, NeuN, Nestin, NF-H and GAP-43) were used to support the fact that the MSCs used *in vivo* were not differentiated into neuro-glial cells. The molecular analysis showed a very small amplification of GFAP gene, absence of amplification of the NF-H and GAP-43 genes, and reasonable amplification of *NeuN*, β-actin, *GAPDH* and Nestin genes. Amplification of a given gene is correlated with its expression seeing that the template DNA is the one generated from mRNA. According to the results of RT-PCR, the molecular analysis showed a small expression of *GFAP* gene and absence of expression of the NF-H and *GAP-43* genes in the expanded MSCs.

### *Biomaterials characterization*

The electrical conductivity achieved for the different tube-guides (PVA, PVA-CNTs and PVA-PPy) was the following: 1.5 ± 0.5 × $10^{-6}$ S/m, 579 ± 0.6 × $10^{-6}$ S/m, and 1837.5 ± 0.7 × $10^{-6}$ S/m, respectively. Therefore these three tube-guide [simple PVA, PVA loaded with 0.05% (%w/v) of COOH-functionalized CNTs and PPy] compositions were chosen for further characterization and for *in vivo* application in the rat sciatic nerve neurotmesis injury model. The thermal characteristics of simple PVA and loaded PVA materials was examined by differential scanning calorimetry and enthalpy of fusion (ΔH) was calculated, and the percentage of crystallinity was near 7.4% for all analyzed nerve guide tubes. Fourier transform infrared spectroscopy analysis the bands identified for PVA loaded with COOH-functionalized CNTs were similar of the bands detected for simple PVA. For PVA loaded with PPy new bands appeared at 1313/cm (C - N stretching vibration in the ring) and 1170/cm (C - H in-plane deformation). Compared with simple PVA the other nerve guide tubes showed a less intensity of the peaks, especially between 2237 and 2380/cm (unpublished data). Considering the XRD analysis previously performed, the broad peak observed at 20° corresponded to a typical diffraction peak of PVA, and it could be also observed in all tube-guides. Near 26° a broad scattering peak appeared for the tube loaded with PPy, and it was an indication of the presence of PPy as supported in literature[46]; (unpublished data).

Both PVA and PVA loaded with PPy when analyzed by SEM exhibited similar surface appearance. On the other hand, the PVA loaded with COOH-functionalized CNTs showed a rougher surface as expected due to the presence of CNTs on PVA matrix, with oriented features. This characteristic was determinant to choose this biomaterial to be associated to the MSCs (PVA-CNTs-MSCs group). The Wettability analysis showed a hydrophilic behavior for the three biomaterials used for tube-guide tested, also the three materials showed negative zeta potential being the PVA the most negative

**Table 1 Static sciatic functional index**

| SSI | | Time | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | T0 | T1 | T2 | T4 | T6 | T8 | T10 | T12 | T14 | T16 | T18 | T20 |
| Group 1: PVA | Mean | -3.13 | -40.52 | -52.76 | -69.60 | -60.30 | -56.59 | -63.51 | -54.89 | -40.25 | -44.03 | -57.34 | -48.23 |
| (*n* = 7) | SD | 24.17 | 19.25 | 17.78 | 9.56 | 19.69 | 13.19 | 8.05 | 11.96 | 13.33 | 9.07 | 21.81 | 7.25 |
| Group 2: PVA-CNTs | Mean | 12.10 | -41.85 | -45.81 | -68.07 | -51.18 | -52.22 | -49.31 | -44.52 | -48.73 | -37.65 | -38.47 | -38.99 |
| (*n* = 7) | SD | 34.10 | 20.82 | 23.21 | 17.00 | 11.41 | 41.96 | 21.11 | 17.62 | 26.31 | 20.78 | 17.97 | 31.11 |
| Group 3: PVA-PPy | Mean | 5.31 | -41.40 | -50.95 | -45.76 | -47.40 | -34.56 | -39.51 | -31.52 | -32.47 | -43.17 | -50.00 | -36.08[b] |
| (*n* = 7) | SD | 17.51 | 17.52 | 23.99 | 29.92 | 22.53 | 14.00 | 25.05 | 15.10 | 20.78 | 25.52 | 11.77 | 16.09 |
| Group 4: Graft | Mean | -6.29 | -56.46 | -52.18 | -55.41 | -63.81 | -59.79 | -75.56 | -53.15 | -64.53 | -67.22 | -57.95 | -52.07 |
| (*n* = 4) | SD | 1.59 | 28.10 | 27.54 | 27.99 | 19.06 | 11.00 | 10.45 | 15.44 | 12.14 | 12.17 | 8.31 | 9.76 |
| Group 5: End-to-End | Mean | -5.78 | -44.21 | -33.72 | -58.28 | -73.92 | -70.22 | -52.47 | -68.04 | -41.91 | -46.56 | -43.04 | -59.97 |
| (*n* = 5) | SD | 12.91 | 15.34 | 15.72 | 22.25 | 6.82 | 6.44 | 27.43 | 11.02 | 24.55 | 16.79 | 12.31 | 2.64 |
| Group 6: PVA-CNTs-MSCs | Mean | 3.46 | -31.48 | -23.75 | -28.57 | -39.97 | -40.30 | -62.32 | -34.71 | -26.28 | -42.87 | -37.54 | -36.16[b] |
| (*n* = 7) | SD | 12.35 | 21.89 | 19.70 | 9.96 | 9.20 | 21.72 | 18.07 | 16.44 | 15.31 | 25.75 | 10.06 | 14.87 |

Static sciatic functional index (SSI) measured pre-operatively (week-0), and every week after the surgical procedure until week-20. An index score of 0 is considered normal and an index of 100 indicates total impairment. The measurements of the toe spread (TS), and the intermediary TS, were taken from the experimental (E) and normal (N) sides. Results are presented as mean and SD. *n* corresponds to the number of rats within the experimental group. PVA: Polyvinyl alcohol; CNTs: Carbon nanotubes; PPy: Polypyrrole; MSCs: Mesenchymal stem cells. [b]$P < 0.01$ *vs* Graft.

surface (-4.97 mV) (Figure 2).

### *Functional analysis during the healing period of 20 wk*

**SFI and SSI analysis:** A few SSI data were missing due to inability to collect visible fingerprints as it was previously referred. As a result of the limited number of animals and the limitations of repeated measures analysis, missing SSI values were replaced using interpolation. Nine of a total of 275 SSI values were interpolated with only one animal having two missing values replace using the linear interpolation procedure. Also because of missing values, the 2-wk time point was not considered for statistical analysis. Sciatic nerve neurotmesis caused a steep decrease in SSI scores in every experimental group and therefore a significant effect of time could be found [$F(10, 200) = 20.445$; $P < 0.001$]. SSI scores remained negative and with little changes throughout the time following sciatic nerve neurotmesis and independently from sciatic nerve treatment, leading to a non-significant time *vs* treatment interaction effect [$F(40, 190) = 279.581$; $P < 0.651$]. However, there was significant differences in the extent of SSI scores' decrease between the experimental groups [$F(4, 20) = 5.848$; $P < 0.01$], with PVA-PPy tube-guides ($P < 0.01$) and PVA-CNTs-MSCs ($P < 0.01$) groups showing less severe SSI scores compared with Graft group (Table 1).

The motor performance by SFI was not possible to perform due to autotomy observed in all treated animals.

**Evaluation of motor performance (EPT) and nociceptive function (WRL):** In the weeks following sciatic nerve neurotmesis there was a large increase in the time latency to withdraw the paw in response to the thermal stimulus, with most animals reaching the cut-off time of 12 s and leading to a significant effect of time [$F(11, 341) = 17.944$; $P < 0.001$]. A mild improvement in latency times occurred over the weeks of recovery at a rate that was similar between the experimental groups so that no significant interaction effect existed [$F(55, 341) = 5.727$; $P < 0.503$]. According to ANOVA results, differences between treated groups (PVA, PVA-CNTs, PVA-PPy, PVA-CNTs-MSCs, Graft and End-to-End) in the severity of changes in WRL times existed [$F(5, 31) = 2.942$; $P < 0.05$], but these differences were not large enough to be detected by the pairwise comparisons (Table 2).

The motor performance by EPT was not possible to perform due to autotomy observed in all treated animals.

**Ankle joint kinematics:** Measures of ankle joint angle at the four selected instants of the stance phase (IC, OT, HR and TO) were collected at the end of 20-wk recovery period. This analysis was carried out only for biomaterial (PVA, PVA-CNTs and PVA-PPy groups, $n = 7$) and biomaterial plus MSCs-treated animals (PVA-CNTs-MSCs group, $n = 7$). Ankle joint angle values were similar across the experimental groups at the times of IC, OT and HR ($P$ = non-significant). However, at TO less acute ankle joint angles were seen for PVA-CNTs-MSCs group, compared with PVA-PPy group ($P < 0.01$) and with PVA-CNTs group showing similar trend also compared with PVA-PPy group ($P = 0.051$). Such less acute ankle joint angles might suggest improved ankle muscles function during the push off phase of the rat's gait cycle (Table 3 and Figure 4).

### *Morphological analysis*

**Muscle results:** After the healing period of 20 wk, TA muscle of all treated rats from the experimental groups (PVA, PVA-CNTs, PVA-PPy, PVA-CNTs-MSCs, Graft and End-to-End) was collect for histological analysis and morphometry to evaluate the secondary neurogenic muscle atrophy associated to neurotmesis injury. It was also evaluated the muscle healing process that followed the sciatic nerve regeneration where the scaffold

**Table 2 Withdrawal reflex latency**

| WRL | | Time | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | T0 | T1 | T2 | T4 | T6 | T8 | T10 | T12 | T14 | T16 | T18 | T20 |
| Group 1: PVA | Mean | 3.71 | 10.95 | 10.81 | 10.68 | 9.74 | 10.23 | 9.85 | 8.15 | 7.78 | 9.99 | 7.88 | 6.98[a] |
| ($n$ = 7) | SD | 0.93 | 1.45 | 2.04 | 2.26 | 2.89 | 2.50 | 2.81 | 2.70 | 3.50 | 2.49 | 3.72 | 3.72 |
| Group 2: PVA-CNTs | Mean | 5.36 | 9.91 | 8.98 | 7.50 | 8.40 | 9.48 | 9.51 | 6.92 | 6.60 | 8.43 | 5.88 | 5.83[a] |
| ($n$ = 7) | SD | 1.50 | 3.57 | 3.65 | 3.36 | 3.22 | 3.41 | 3.01 | 3.49 | 3.41 | 3.40 | 3.09 | 2.80 |
| Group 3: PVA-PPy | Mean | 4.86 | 11.42 | 9.37 | 7.10 | 7.85 | 8.82 | 9.72 | 6.87 | 7.02 | 7.09 | 6.06 | 7.02[a] |
| ($n$ = 7) | SD | 1.69 | 1.54 | 3.31 | 2.51 | 3.40 | 2.53 | 2.35 | 3.50 | 3.92 | 3.23 | 4.43 | 3.89 |
| Group 4: Graft | Mean | 4.53 | 12.00 | 10.14 | 11.17 | 11.50 | 10.67 | 10.83 | 11.16 | 9.81 | 10.46 | 9.92 | 9.68[a] |
| ($n$ = 4) | SD | 1.14 | 0.00 | 3.72 | 1.66 | 1.01 | 2.65 | 2.35 | 1.27 | 2.84 | 1.78 | 1.82 | 1.88 |
| Group 5: End-to-End | Mean | 4.28 | 12.00 | 11.85 | 12.00 | 10.22 | 10.45 | 8.78 | 10.48 | 9.26 | 8.57 | 9.82 | 7.50 |
| ($n$ = 5) | SD | 0.86 | 0.00 | 0.34 | 0.00 | 2.50 | 2.12 | 2.22 | 2.86 | 3.07 | 0.98 | 2.02 | 0.87 |
| Group 6: PVA-CNTs-MSCs | Mean | 3.93 | 12.00 | 8.89 | 9.23 | 9.30 | 9.90 | 7.65 | 7.41 | 10.06 | 8.33 | 8.28 | 8.02[a] |
| ($n$ = 7) | SD | 0.86 | 0.00 | 2.36 | 1.63 | 2.72 | 2.66 | 2.51 | 2.33 | 1.85 | 2.86 | 2.34 | 1.20 |

Values in seconds were obtained performing withdrawal reflex latency (WRL) test to evaluate the nociceptive function. This test has been performed pre-operatively (week-0), at week 1, week 2 and every 2 week after the surgical procedure until week-20, when the animals were sacrificed for morphological analysis. Results are presented as mean and standard error of the mean (SD). $n$ corresponds to the number of animals within each experimental group. PVA: Polyvinyl alcohol; CNTs: Carbon nanotubes; PPy: Polypyrrole; MSCs: Mesenchymal stem cells. [a]$P < 0.05$ *vs* End-to-End.

**Table 3 Ankle kinematics**

| Temporal events | Group | Week 20 |
|---|---|---|
| IC | Group 1: PVA | -3.23 ± 20.65 |
| | Group 2: PVA-CNTs | -8.32 ± 17.36 |
| | Group 3: PVA + PPy | 0.82 ± 26.33 |
| | Group 6: PVA-MSCs | -13.68 ± 8.57 |
| OT | Group 1: PVA | 28.30 ± 19.52 |
| | Group 2: PVA-CNTs | 27.67 ± 12.91 |
| | Group 3: PVA + PPy | 17.66 ± 13.20 |
| | Group 6: PVA-MSCs | 11.43 ± 9.69 |
| HR | Group 1: PVA | 46.67 ± 14.81 |
| | Group 2: PVA-CNTs | 47.69 ± 11.19 |
| | Group 3: PVA + PPy | 36.46 ± 10.14 |
| | Group 6: PVA-MSCs | 34.07 ± 6.85 |
| TO | Group 1: PVA | 33.99 ± 9.57 |
| | Group 2: PVA-CNTs | 27.01 ± 10.99 |
| | Group 3: PVA + PPy | 43.90 ± 8.76 |
| | Group 6: PVA-MSCs | 20.56 ± 11.31 |

Values were obtained performing video analysis at 20-wk of the healing period. Results are presented as mean and standard deviation (SD) ($n$ = 7). For each step cycle the following time points were identified: Initial contact (IC), opposite toe off (OT), heel rise (HR) and toe-off (TO). PVA: Polyvinyl alcohol; CNTs: Carbon nanotubes; PPy: Polypyrrole.

was applied. It was possible to observe by muscle morphometry that there was a significant difference ($P < 0.05$) in terms of the increase in mean fiber size between some of the treatment (PVA-PPy and PVA-CNTs) groups and the Graft group. In fact, in the PVA-PPy group there was a 25% increase in terms of average fiber area and a 13% increase in terms of the "minimal Feret's diameter", when compared to the Graft group. Whereas in the PVA-CNTs treatment group there was a 42% increase in terms of average fiber area and a 21% increase in term of the "minimal Feret's diameter", when compared to the Graft group. Although both these two treatment groups (PVA-PPy and PVA-CNTs) exhibited significant improvement in fiber size, approaching the values for normal muscles (collected from rats with no sciatic nerve neurotmesis lesion), both of them were still significantly different ($P < 0.05$) from the control group (without lesion) at 20 wk after lesion. PVA treatment without any coating (PVA group) revealed no regeneration benefit since the mean fiber size was lower (with significant difference in terms of "minimal Feret's diameter"; $P < 0.05$) when compared to the Graft group (Figure 5). However, unexpected results were observed with PVA-CNTs-MSCs, which showed the worst results in terms of muscular atrophy after denervation, results not correlated with functional analysis (considering mostly kinematics gait analysis and SSI) (Figure 5). The decreased fiber size observed in these animals' TA muscles was coincident with the smaller sized muscles that were collected at 20 wk after surgery macroscopically evaluated during the collection for histological evaluation. Histologically it was also possible to detect a considerable amount of necrosis with delayed muscle regeneration in this group's muscles (Figure 6).

**Histomorphometry of the regenerated sciatic nerve:** Histological analysis showed that nerve fiber regeneration occurred in all reconstructed nerves. In comparison to controls (sciatic nerve without neurotmesis injuries, data not shown)[3], in all repaired nerves regenerated fibers showed small axons with thin myelin sheaths and microfasciculation (Figure 7). Microfasciculation was more evident in the PVA-CNTs-MSCs repaired group (Figure 7 and Table 4). Also, stereological analysis disclosed a significantly ($P < 0.05$) larger axon diameter, but not fiber diameter, in the regenerated nerves from PVA-PPy group compared with those from PVA-CNT-MSC group (Table 4). On the other hand, as it was previously reported[3,25], the application of MSCs-based cell therapy to neurotmesis nerve injuries resulted in a significantly increased myelin thickness (M), ratio myelin thickness/axon diameter (M/d) and ratio axon diameter/fiber diamenter (d/D; g-ratio) when compared to PVA, PVA-CNTs, PVA-PPy groups ($P < 0.05$). The results clearly demonstrate a

**Table 4 Stereological quantitative assessment**

| Group | | Density | Total number | Axon diameter (d) | Fiber diameter (D) | Myelin thickness (M) | M/d | D/d | d/D (g-ratio) | Area ($mm^2$) |
|---|---|---|---|---|---|---|---|---|---|---|
| Group 1: PVA | Mean | 42872 | 16596 | 2.95 | 3.56 | 0.31[a] | 0.11[a] | 1.23 | 0.82[a] | 0.3862 |
| ($n$ = 7) | SD | 4007 | 1741 | 0.25 | 0.28 | 0.03 | 0.01 | 0.02 | 0.01 | 0.0772 |
| Group 2: PVA-CNTs | Mean | 36261 | 16049 | 2.98 | 3.71 | 0.37[a] | 0.14[a] | 1.28 | 0.79[a] | 0.4455 |
| ($n$ = 7) | SD | 4267 | 1172 | 0.25 | 0.27 | 0.01 | 0.01 | 0.03 | 0.01 | 0.1856 |
| Group 3: PVA-PPy | Mean | 40289 | 22588 | 3.03[a] | 3.70 | 0.33[a] | 0.12[a] | 1.24 | 0.81[a] | 0.5720 |
| ($n$ = 7) | SD | 2686 | 3157 | 0.21 | 0.23 | 0.02 | 0.01 | 0.01 | 0.00 | 0.2565 |
| Group 4: Graft | Mean | 37041 | 21939 | 2.58 | 3.56 | 0.49 | 0.21 | 1.41 | 0.71 | 0.5936 |
| ($n$ = 4) | SD | 1214 | 1302 | 0.06 | 0.08 | 0.02 | 0.01 | 0.02 | 0.01 | 0.0694 |
| Group 5: End-to-end | Mean | 38762 | 22729 | 2.65 | 3.58 | 0.47 | 0.19 | 1.38 | 0.73 | 0.5990 |
| ($n$ = 5) | SD | 1524 | 2308 | 0.14 | 0.16 | 0.01 | 0.01 | 0.01 | 0.01 | 0.2082 |
| Group 6: PVA-CNTs-MSCs | Mean | 43373 | 25731 | 2.44 | 3.34 | 0.45[c,e] | 0.21[c,e] | 1.41 | 0.72[c,e] | 0.5942 |
| ($n$ = 7) | SD | 3881 | 3386 | 0.20 | 0.21 | 0.06 | 0.04 | 0.08 | 0.01 | 0.1940 |

Density, total number, axon diameter (d), fiber diameter (D), myelin thickness (M), ratio myelin thickness and axon diameter (M/d), ratio fiber diameter and axon diameter (D/d), ratio axon diameter and fiber diameter (d/D, g-ratio) of regenerated sciatic nerve fibers at week-20 after neurotmesis. Values are presented as mean ± SD. $n$ corresponds to the number of animals within each experimental group. PVA: Polyvinyl alcohol; CNTs: Carbon nanotubes; PPy: Polypyrrole. [a]$P < 0.05$ *vs* PVA-CNT-MSC; [c]$P > 0.05$ *vs* End-to-End; [e]$P > 0.05$ *vs* Graft.

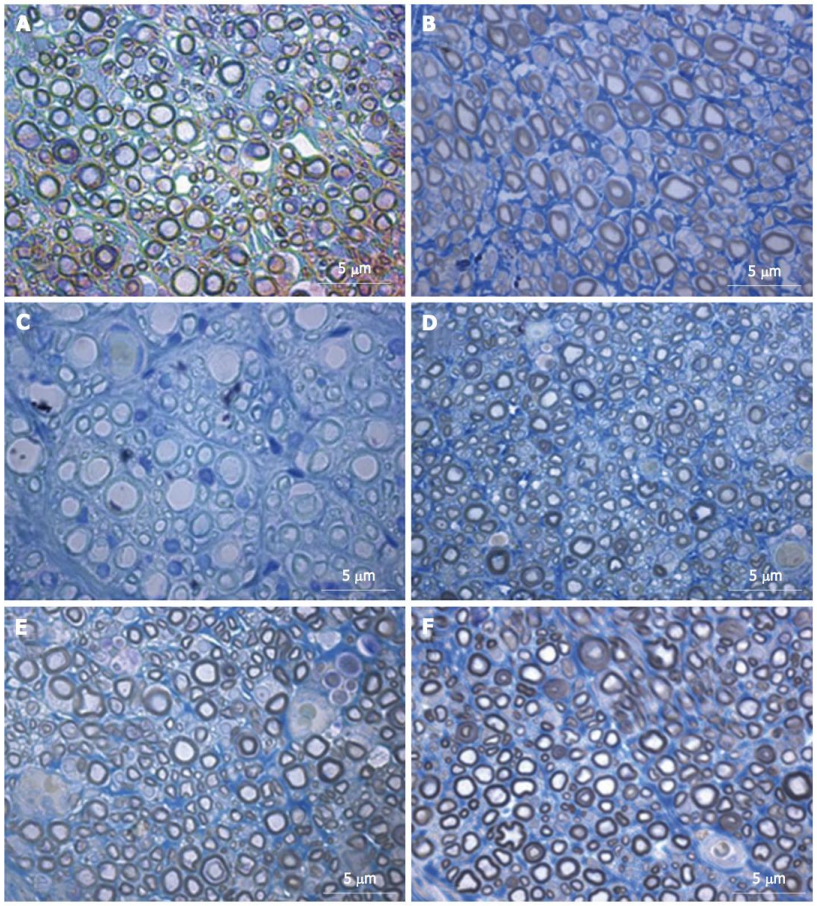


**Figure 7 Histological appearance of regenerated nerve fiber in the different groups of neurotmesis.** A: PVA; B: PVA-CNTs; C: PVA-PPy; D: PVA-CNTs-MSCs; E: Graft; F: End-to-end suture (magnification: 1000 ×). PVA: Polyvinyl alcohol; CNTs: Carbon nanotubes; PPy: Polypyrrole; MSCs: Mesenchymal stem cells.

synergistic positive effect on regenerated nerve fibers resulting from combined use of MSCs with the PVA-CNTs tube-guides. Also, in the PVA-CNTs-MSCs group these three stereological parameters (M, M/d, and g-ratio) were not significantly different from the values obtained from End-to-End and Graft groups (Table 4). The total number of nerve fibers and nerve fibers density were similar in all groups.

**Histology of internal organs:** At the microscopic exam, no alterations were observed. The histology sections of all organs confirmed the absence of inflammation, cell degeneration, necrosis and fibrosis. No signs of nanotubes, neither carbon deposits were detected in all organs.

## DISCUSSION

An organ re-innervation and functional recovery after nerve injury has been intensively studied worldwide and regenerative medicine methods including cellular systems and new biomaterials concerning the peripheral nerve have been developed. The involved mechanisms in the regeneration process and the ideal scaffold have not been discovered yet. Peripheral nerve might regenerate after some types of injury like axonotmesis but it is difficult to achieve functional recovery after neurotmesis, especially when there is loss of nerve tissue and a gap is created. In the peripheral nervous system, nerves after axonotmesis injuries can regenerate, without any treatment but in most clinical cases a muscle regional neurogenic atrophy occurs. When neurotmesis injuries occur, the nerves must be surgically treated by direct end-to-end suture[33,47,48], using appropriate micro-surgery techniques and suturing material. Nowadays most tissue engineered nerve grafts are composed of a neural scaffold prepared with a variety of biomaterials, and surgically applied in neurotmesis injuries with loss of nervous tissue where the direct end-to-end suture is not possible creating tension in the suture line and compromising the nerve regeneration[9]. The introduction of cell therapies should be delivered by appropriate vehicles promoting an important biochemical and physical cue. MSCs represent an appealing source of adult stem cells for cell therapy and tissue engineering including peripheral nerve. Because MSCs are present in low percentage in the adult BM and also because of some of the disadvantages previously listed, alternative sources have been studied, like the UCT (also called Wharton's jelly). MSCs were defined by the ISCT in 2006, and since that time it is considered that MSCs must adhere to plastic; have a specific profile for the specific surface markers (positive for CD73, CD90, and CD105, and negative for CD14, CD19, CD34, CD45 and HLA-DR). Other important characteristic is that MSCs are also able to undergo tri-lineage differentiation into adipocytes, chondrocytes and osteoblasts[19]. I*n vivo* and *in vitro* studies referred that MSCs have the capacity to induce the regeneration of cartilage, bone, tendon, and meniscus[18]. The MSCs are also able to differentiate into neuro-glial cells, which have been studied *in vitro* and *in vivo* using animal models, by our research group[25]. Children and adult clinical trials have been performed where MSCs were used to treat a wide range of pathologies, including neurological disorders, PNS and CNS injuries (access www.clinicaltrials.gov). In fact, nowadays, the cryopreservation of UCB, and UCT is performed worldwide in private and public cord blood banks. Furthermore, the MSCs from the UCT and hematopoietic stem cells from the UCB have positive clinical outcomes not only in hematologic malignancy patients, where the MSCs work as a co-adjuvant for the hematopoietic transplant success, but also in PNS injuries and neurologic disorders. For that reason, the crucial role of MSCs in tissue renewal and regeneration has been well established for several limiting pathologies, not treated by the traditional therapeutic approaches[49]. The *in vivo* application of MSCs performed by the authors was intended to improve the regeneration process in the rat sciatic nerve after a neurotmesis injury which was surgically reconstructed using electric conductive tube-guides of PVA-CNTs composition. At that moment there are several nerve tube-guides in the market, however they still have limitations and the functional outcome of the patients is still not complete. In this work the previously developed tube-guides with high electrical conductivity for nerve regeneration (unpublished data) were used *in vivo* using the rat sciatic nerve neurotmesis injury model. A matrix of PVA was used loaded with the following electrical conductive materials: COOH-functionalized multiwall carbon nanotubes (MWCNTs) and PPy PVA-CNTs and PVA-PPy tube, guides, respectively. The tubes production was carried out by a freezing/thawing process (physical cross-linking) and a final annealing treatment. After producing the tube-guides, the physicochemical characterization was performed. The most interesting results were achieved by loading PVA with 0.05% of PPy or COOH-functionalized CNTs, by combining the electrical conductivity of CNTs and PPy with the biocompatibility of PVA matrix, which seems to have potential to be used in peripheral nerve regeneration. For that reason, these two biomaterials were chosen to proceed for *in vivo* pre-clinical trials using the rat sciatic model. As a matter of fact, the microscopic exam performed to lung, heart, kidneys, liver and spleen collected after animal euthanasia, confirmed the absence of inflammation, cell degeneration, necrosis and fibrosis, and no signs of nanotubes, neither carbon deposits were detected in all organs, confirming the biocompatibility of these biomaterials loaded with CNTs. The evaluation of cytocompatibility was performed by measuring the $[Ca^{2+}]_i$ in Fura-2-loaded MSCs cells by using dual wavelength spectrofluorometry, sub-cultured over *PVA* discs, over PVA loaded with carbon nanotubes (PVA-CNTs) and PVA loaded with PPy (PVA-PPy) discs of 10 mm diameter, as previously described[3,25,27]. Results correspond to $[Ca^{2+}]_i$ from viable MSCs that did not begin the apoptosis

process, confirming the MSCs ability to expand and viability when associated to PVA, PVA-PPy and PVA-CNTs tube-guides.

The MSCs isolated from the WJ used as cell-based therapy for promoting nerve regeneration when associated to electric conductive tube-guides was previously *in vitro* validated concerning flow cytometry profile, RT-PCR, and cytogenetic analysis. MSCs were expanded to P5-P6 where the culture appeared homogeneous and cells presented their typical fusiform, fibroblast-like, morphology (Figure 1). Also, the flow cytometry analysis confirmed their profile according to ISCT[19] and the karyotype demonstrated that no structural alterations were present demonstrating absence of neoplastic characteristics in these cells, as well as chromosomal stability to the cell culture procedures. RT-PCR and qPCR targeting specific genes expressed by the MSCs *in vivo* applied was performed to certify that the MSCs used *in vivo* were not differentiated into neuro-glial cells. For the MSCs, the molecular analysis showed a very small amplification of *GFAP* gene, absence of amplification of the *NF-H* and *GAP-43* genes, and reasonable amplification of NeuN, β-actin, *GAPDH* and Nestin genes, confirming that MSCs used *in vivo* were undifferentiated and not neuro-glial differentiated. In fact, the small detection of the *GFAP* gene expression may be due to the high sensitivity of the molecular tests in comparison with immunocytochemistry tests previously performed using undifferentiated and neuro-glial type differentiated MSCs[3,18]. Moreover, the expression of the remaining genes, NeuN, β-actin, GAPDH and Nestin was also observed in undifferentiated MSCs. The expression of the housekeeping genes, β-actin and GAPDH, is expected to occur. As per the NeuN and Nestin gene, the observation of its expression in undifferentiated MSCs is not new; Bertani *et al*[50] showed that naïve MSCs express at a constitutive level NeuN gene, which increases when these cells are chemically induced to differentiate to pre-neuronal cells. Furthermore, compared gene expression profiles before and after MSCs induction for a number of germ layers, and observed that even before neuronal induction, MSCs population and clonal lines expressed a mixture of mesodermal, germinal, endodermal and ectodermal genes, including several whose expression was thought to be restricted to neuronal cells[51]. Molecular analysis previously performed[18] on these same genetic markers over the differentiated MSCs showed an increase in the expression of *GFAP*, *NF-H* and *GAP-43* genes. These genes were not expressed, or expressed at very low levels, in the undifferentiated MSCs transcriptome. Overall, these results support the fact that the MSCs used *in vivo* were not differentiated but presented a neuro-glial potential for differentiation confirming previous results where the same MSCs undifferentiated and *in vitro* differentiated into neuro-glial cells were tested in axonotmesis and neurotmesis injuries associated to other biomaterials namely poly(DL-lactide-ε-caprolactone), hybrid chitosan and collagen[3,25,31].

The rat sciatic nerve model has been widely used in experiments on peripheral nerve regeneration and it has been demonstrated that it is reliable and reproducible model, but research on peripheral nerve injury needs to include both functional and morphological analysis[52]. Since it is not generally agreed which type of evaluation tool is the most useful for evaluation of functional recovery; our research group like others has been using different methods for an overall assessment of nerve function, which has been widely recommended[53]. The sciatic nerve regeneration after neurotmesis studies described by the authors were followed during 20 wk after the surgical procedure based on the previous experimental work[17,29,54,55]. The morphological evaluation together with functional data has been used to assess neural regeneration after induced neurotmesis injuries, but some subjective evaluation, depending on the operator/research analysis is observed[9]. Some methods for evaluation of nerve recovery, like peroxidase and retrograde fluorescent labeling, histomorphometry, and retrograde transport of horseradish[47,56] fail in assessing the functional recovery, which is essential to evaluate the success of a scaffold application[57,58]. The present experimental work includes a variety of independent evaluation tools considering the morphologic and functional recovery, in order to understand and estimate the potential therapeutic benefit of a nerve repair strategy[9]. EPT, WRL, SSI and SFI, have been proven to be valid methods and to give some information to determine functional recovery following sciatic nerve injury, including motor and nociceptive evaluation[59]. The use of biomechanical techniques and rat's gait kinematic evaluation was a progress in documenting functional recovery, largely published by our research group[3,9,17,31,33,35]. Indeed, the use of biomechanical parameters allows an accurate analysis of the sciatic denervation/reinnervation process, permitting to understand the integration of the neural control acting on the ankle and foot muscles, and thus allowing to evaluate the nerve and muscle regeneration after neurogenic muscle atrophy associated to neurotmesis injuries[60,61]. At the end of the 20-wk healing period, it was performed kinematic gait analysis of rats from the groups where it was applied the biomaterial alone or associated to MSCs-based therapies (PVA, PVA-CNTs, PVA-PPy, and PVA-CNTs-MSCs groups) and measures of ankle joint angle at the four selected instants of the stance phase (IC, OT, HR and TO) were obtained (Table 3 and Figure 4). Considering the histological analysis, it was performed stereology of the regenerated sciatic nerves and morphometry analysis of the TA muscles, trying in this way, not only correlate the neurotmesis injury and the secondary neurogenic regional atrophy, but also consider and validate the morphometry of regional muscles (for instance, by biopsy of the injured muscle) as a method to assess functional recovery after peripheral nerve injuries[18,25]. The restoration of locomotor activity following damage of the PNS has emerged as one of the most important and critical clinical problem in

neuroscience and neurosurgery fields. Regional muscle weakness and impairment of joint control and mobility occurs in many patients with peripheral nerve or spinal cord injuries which results in gait disorders only evaluated with accuracy by kinematics analysis. During the past 10-15 years, experimental work has been carried out on rat gait analysis which may significantly contribute in the future for a more precise peripheral nerve research. Indeed, the use of biomechanical parameters permits an integration of the neural control acting on the ankle and foot muscles, relevant for the analysis of the sciatic denervation/reinnervation effects. The biomechanical analysis is very useful and accurate to evaluate different therapeutic approaches, describing high number of kinematic variables including positions, velocities, and accelerations, often using high speed digital cameras[35,40,62,63]. There is high variability between individual joint kinematics and between different animals from the same the same experimental group[64]. The high level of variability observed in normal quadruped walking, affects significantly the precision of joint kinematic measures of functional recovery after nerve injury. It is important to reduce this variability in kinematics analysis to assess functional recovery after neurogenic muscle atrophy and neurotmesis/axonotmesis injuries. One evident advantage is that using a treadmill the operator reduces step-by-step variability in joint kinematics[59] which has the disadvantage of being expensive equipment. Also the possibility of combining kinematic analysis with other data, such as ground reaction forces, should be consider for a more accurate evaluation of nerve regeneration[59,64,65]. Our research group has recently analyzed hip, knee and ankle joint kinematics during recovery of rat sciatic nerve axonotmesis injury, using reflective markers attached to the rat hind-limb to track the motion with infra-red capture cameras, to better assess the rat sciatic nerve model hind-limb joint kinematics during walking. Due to physiological constraints and muscle actions it was observed that different joints have different motion patterns within motion planes, more evident when a 3D segmental kinematic analysis using a 3D reconstruction of the rat hind-limb was performed[9]. This method allowed a more complete segmental kinematic analysis using both planar angles computation (2D) and a 3D reconstruction of the rat hind-limb but unfortunately, in the present experimental work it was only possible to perform a 2D gait analysis[9]. In the present work, 2D biomechanical analysis (sagittal plan) was carried out applying a two-segment model of the ankle joint, adopted from the model firstly developed by Varejão *et al*[40]. For that reason, ankle joint angle values were similar across the experimental groups at the times of IC, OT and HR. However, less acute ankle joint angles observed in PVA-CNTs-MSCs group, compared with PVA-PPy and PVA-CNTs groups might suggest improved ankle muscles function during the push off phase of the rat's gait cycle (Figure 4 and Table 3). It is well known and demonstrated by the scientific bibliography that neuromuscular pathologies are related to important clinical signs or motor deficits that should be observed, qualified, and quantified, only possible with a precise kinematic analysis[65,66]. In the field of peripheral nerve research using the rat sciatic nerve model, an improved walking analysis might include several methods combination like joint kinematics, ground reaction forces and electromyographical data of muscle activity. These methods refinements might be important to differentiate the regenerative potential of different scaffolds used, that are not evident when using the traditional standardized methods previously referred[9,66].

The recent published paper by di Summa *et al*[67] where collagen tube-guides (Neurogen®) were used *in vivo* to promote peripheral nerve regeneration, combined with schwann cells (SCs), it was possible to demonstrate an improved distal stump sprouting[68]. This sprouting was more pronounced in the experimental group where the SCs were derived from BM-MSCs when compared to SCs derived from adipose tissue MSCs (AT-MSCs). On the other hand, no significant differences were observed in proximal regeneration among all the experimental groups. BM-MSCs and AT-MSCs -loaded conduits induced a diffuse sprouting pattern of the axons. On the other hand, the tube-guides loaded with SCs induced an enhanced cone pattern and a typical sprouting along the collagen type I conduits walls, showing improved regenerating results. This observation is important and should be related to results obtained from the innervated muscle morphometry analysis. It should be bear in mind that the sprouting is also evaluated and a constant observation in the histomorphometry analysis of the regenerated peripheral nerve after axonotmesis and neurotmesis lesions, where different reconstruction strategies were tested *in vivo* in the rat model by our research group for the past years[3,25,31,68].

The morphometry and histological analysis of TA muscles collected from rats where the neurotmesis injury was reconstructed with PVA-CNTs-MSCs tube-guides is in fact somehow surprising since in the treatment groups presented in a previous published study (Gärtner *et al*[3], 2014), no adverse effect was noticed when MSCs isolated from UC WJ were used. The TA muscles from PVA-CNTs-MSCs group presented the worst results in terms of muscular atrophy after denervation, results not correlated with functional analysis (considering mostly kinematics gait analysis and SSI) (Figure 5). The decreased fiber size observed in these TA muscles was coincident with the smaller sized muscles that were collected at 20 wk, macroscopically evaluated during the collection for histological evaluation. Histologically it was also possible to detect a considerable amount of necrosis with delayed muscle regeneration in this group's TA muscles (Figure 6). Some toxic metabolite resulting from the interaction between the vehicle where the MSCs are suspended (PBS) which has calcium and magnesium, and PVA-CNTs tube-guide, could explain the histological results. These results may occur, not because there is a deleterious effect of the cellular

system, but because the vehicle is not appropriate to the cells microenvironment. As a matter of fact these MSCs were previously tested associated to Floseal® and some local nerve necrosis could be observed by histological analysis. On the other hand, the myelin sheath of the MSCs-treated regenerated nerves was thicker, suggesting that MSCs might exert their positive effects on SCs, the key element in Wallerian degeneration and consequent axonal regeneration[3]. From the data obtained from the stereology analysis of PVA, PVA-CNTs, PVA-PPy, Graft, End-to-End and PVA-CNTs-MSCs groups, it was also demonstrated a synergistic positive effect on regenerated nerve fibers resulting from combined use of MSCs with the PVA-CNTs tube-guides, where PVA-CNTs-MSCs presented higher myelin thickness (M), ratio myelin thickness/axon diameter (M/d) and ratio axon diameter/fiber diameter (d/D; g-ratio) when compared to PVA, PVA-CNTs, PVA-PPy groups (Table 4). Also, these three stereologic parameters (M, M7d, and g-ratio) measured in PVA-CNTs-MSCs regenerated nerves, were not significantly different from the values obtained from End-to-End and Graft groups. In conclusion, as it was previously reported[3,25], in the MSCs-based cell therapy applied to neurotmesis nerve injuries, it is observed a significantly higher myelin thickness, and similar to Graft and End-to-End regenerated nerves, which are considered the Gold Standards in nerve neurosurgery. These results clearly are consisted with functional data, from SSI and kinematic gait analysis. The PVA-CNTs and PVA-PPy groups presented significantly higher axon diameters (d) and fiber diameters (D), suggesting also the positive effect of these electric conductive materials on axon regeneration and reestablishment of the neuro-muscular junction in agreement with the TA muscle morphometry analysis.

In conclusion, the results revealed that treatment with MSCs associated to PVA-CNTs tube-guides induced an increased number of regenerated fibers and thickening of the myelin sheet. Functional and kinematics analysis has revealed positive synergistic effects brought by MSCs and PVA-CNTs. The PVA-CNTs and PVA-PPy are promising scaffolds with electric conductive properties, bio and cytocompatible, that might prevent in some degree the secondary neurogenic muscular atrophy by improving the reestablishment of the neuro-muscular junction. Since the present studies were carried out in rats with human MSCs, it is important to continue these studies to evaluate the therapeutic potential of these MSCs in human nerve injuries. However, the results discussed herein show a promising effect of MSCs and PVA-CNTs tube-guides in in neurodegenerative diseases that are typified by demyelination since this scaffold induced myelin production in surgically reconstructed nerves after a neurotmesis injury.

## COMMENTS

### Background

The sought for effective new therapeutic strategies for improving peripheral nerve regeneration represents one of the hot topics in biomedicine because of the high number of lesions affecting peripheral nerves (much higher than lesions to the spinal cord).

### Research frontiers

In this study, the authors have tested *in vitro* and *in vivo* mesenchymal stem cells (MSCs) derived from the umbilical cord matrix [Wharton's jelly (WJ)] associated to electric conductive tube-guides developed by their research group, focusing on its effect in promoting nerve regeneration in the rat neurotmesis model. In particular, *in vivo* assessment of nerve regeneration and functional recovery was carried out using a comprehensive battery of complementary methods of analysis that the authors have developed along their previous experience in the study of nerve repair and regeneration and which include behavioral analysis of motor and nociceptive function, kinematic analysis using high speed cameras and histological analysis of nerve fiber regeneration.

### Innovations and breakthroughs

The results of this study open interesting perspectives for the clinical application of biodegradable membranes in peripheral nerve reconstruction associated to MSCs isolated from the umbilical cord matrix. Interestingly, these cells, which are major histocompatibility complex class Ⅱ negative, not only express both an immune-privileged and immune-modulatory phenotype, but their major histocompatibility complex class Ⅰ expression levels can also be manipulated, making them a potential cell source for MSC-based therapies. In addition, these cells represent a non controversial source of primitive mesenchymal progenitor cells that can be harvested after birth, cryogenically stored, thawed, and expanded for therapeutic uses.

### Applications

The results revealed that polyvinyl alcohol (PVA)-carbon nanotubes (CNTs) and PVA-polypyrrole (PPy) are promising scaffolds with electric conductive properties, bio and cytocompatible, that might prevent in some degree the secondary neurogenic muscular atrophy by improving the reestablishment of the neuro-muscular junction. Also, a promising effect of MSCs and PVA-CNTs tube-guides in promoting myelin production in surgically reconstructed nerves after a neurotmesis injury was demonstrated. A new gateway it therefore opened for using these cells in neurodegenerative diseases that are typified by demyelination.

### Terminology

Neurotmesis (in Greek tmesis signifies "to cut") is part of Seddon's classification used to classify nerve damage. It is the most serious nerve injury. In this type of injury, both the nerve and the nerve sheath are disrupted. Nowadays, when a nerve is damage and there is a neurotmesis injury with a gap, it can be repaired using a nerve tube-guide, whenever it is not possible to perform an end-to-end suture without tension or there isn't a graft available. This work focused on the development of electrical conductive nerve tube-guides, which was achieved by loading PVA with PPy or COOH-functionalized CNTs and their *in vivo* application in the rat sciatic nerve neurotmesis injury model. The inclusion of CNTs and PPy brought a significant increase of electrical conductivity of the simple PVA tube-guide. MSCs as defined by the international society for cellular therapy in 2006, are cells characterized by: (1) their capacity to adhere to plastic; and (2) expression of specific surface markers, namely, CD73, CD90, and CD105, and no expression of CD14, CD19, CD34, CD45 and human leukocyte antigen-DR. Additionally, MSCs are able to undergo tri-lineage differentiation into adipocytes, chondrocytes and osteoblasts.

### Peer-review

The article is very interesting. The experimental part is very well designed.

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## *2.3.* Outras aplicações do PVA – *Cell therapy with human MSCs isolated from the umbilical cord Wharton Jelly associated to a PVA Membrane in the treatment of chronic wounds.*

Durante o período em que decorreram os estudos no âmbito deste Doutoramento, foi mantida a atividade clínica na área da cirurgia de animais de companhia no UPVet – Hospital Veterinário da Universidade do Porto. No decurso dessa atividade clínica surgiram 2 casos clínicos de cães, que considerámos potencialmente benéfica a utilização do PVA assim como da aplicação de MSCs, em feridas crónicas. Esse trabalho clínico resultou no seguinte artigo científico:

**Ribeiro J**, Pereira T, Amorim I, Caseiro AR, Lopes M.A, Lima J, Gartner A, Santos JD, Bártolo PJ, Rodrigues JM, Maurício AC, Luís AL (2014). **Cell therapy with human MSCs isolated from the umbilical cord Wharton jelly associated to a PVA membrane in the treatment of chronic skin wounds**. *International Journal of Medical Sciences*, *11*(10), 979.

Outros casos foram igualmente realizados, no entanto encontram-se em fase de avaliação, pelo que se decidiu não os incluir nesta dissertação.

**Cell therapy with human MSCs isolated from the umbilical cord Wharton Jelly associated to a PVA Membrane in the treatment of chronic wounds**

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Research Paper

# Cell Therapy with Human MSCs Isolated from the Umbilical Cord Wharton Jelly Associated to a PVA Membrane in the Treatment of Chronic Skin Wounds

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## Abstract

The healing process of the skin is a dynamic procedure mediated through a complex feedback of growth factors secreted by a variety of cells types. Despite the most recent advances in wound healing management and surgical procedures, these techniques still fail up to 50%, so cellular therapies involving mesenchymal stem cells (MSCs) are nowadays a promising treatment of skin ulcers which are a cause of high morbidity. The MSCs modulate the inflammatory local response and induce cell replacing, by a paracrine mode of action, being an important cell therapy for the impaired wound healing. The local application of human MSCs (hMSCs) isolated from the umbilical cord Wharton's jelly together with a poly(vinyl alcohol) hydrogel (PVA) membrane, was tested to promote wound healing in two dogs that were referred for clinical examination at UPVET Hospital, showing non-healing large skin lesions by the standard treatments. The wounds were infiltrated with 1000 cells/µl hMSCs in a total volume of 100 µl per $cm^2$ of lesion area. A PVA membrane was applied to completely cover the wound to prevent its dehydration. Both animals after the treatment demonstrated a significant progress in skin regeneration with decreased extent of ulcerated areas confirmed by histological analysis. The use of Wharton's jelly MSCs associated with a PVA membrane showed promising clinical results for future application in the treatment of chronic wounds in companion animals and humans.

Key words: Chronic wounds, mesenchymal stem cells, Wharton's jelly, PVA hydrogel, histology.

## Introduction

Proper cutaneous wound repair requires a good coordination of inflammation, neovascularisation, extracellular matrix (ECM) formation and epithelialisation. When skin is severely damaged, the ability to repair itself is limited resulting in hypertrophic scars, so-called keloid scar, and consequent non-healing, or ulceration, or excessive scar contraction of the wound. Traditional therapies for treatment of chronic wound include surgical debridement, minimization of local bacterial multiplication; pressure off-loading, negative-pressure therapy, skin grafting and reconstructive tissue flaps. Despite the most recent advances in wound healing management and surgical procedures, these techniques still fail up to 50% [1]. The difficulty of the healing process of chronic wounds might be due to local ischemia, reperfusion injury, bacterial infection, or aging resulting in chronic inflammation [2]. Also in non-healing wounds the cell pool is intrinsically impaired and might demonstrate increased senescence and decreased response to growth factors [3, 4]. MSCs present high plasticity, proliferative and differentiation capacity together with important immunosuppressive properties and low immunogenicity due to a decreased Human Leucocyte Antigen (HLA) class II expression [5-9], representing a promising cellular system to improve wound healing.

There are many studies involving mice and humans which have consistently demonstrated enhanced wound repair following treatment by infiltration or lesion local application, with bone marrow-derived MSCs [10, 11] or MSCs from other sources, like the umbilical cord blood (UCB) [12] and adipose tissue [13] Analysis of the mechanical properties of treated wounds revealed that administration of MSCs not only accelerated wound closure but also enhanced wound repair quality, resulting in repaired tissue with increase tensile strength. This effect is thought to be secondary to an increased in collagen composition within the repaired tissue [14].

MSCs isolated from the umbilical cord matrix (Wharton's jelly) have many advantages, such as, shorter population doubling time; can be easily cultured in plastic flasks, are well tolerated by the receptor immune system, so transplantation into non-immune-suppressed animals does not induce acute rejection, have anticancer properties [7] and most important are not tumorigenic [15, 16]. For these reasons we believe that cellular therapy using MSCs from the Wharton´s jelly in chronic wounds treatment is a promising field of investigation with great interest in Regenerative Medicine.

The MSCs delivery method is also very important and some authors used subcutaneous injection, systemic injection, intradermal injection and topical applications or combinations of these routes [17, 18]. The clinical effectiveness of MSCs-based therapy is dependent on the number of cells delivered and survival, so it is important to optimize the delivery procedure. Falanga and collaborators utilized a fibrin spray system to topically administer autologous MSCs to non-healing lower extremity wounds in human subjects detecting considerable clinical improvements [18,19]. Hydrogels, as new generation synthetic biomaterials, are being developed at a fast speed to be used as three-dimensional extracellular microenvironment in order to simulate the regulatory characteristics of natural ECMs and ECM-bound growth factors, both for therapeutic applications and basic biology studies. Although modern synthetic biomaterials represent oversimplified mimics of natural ECMs lacking the essential natural temporal and spatial complexity, a growing symbiosis of engineered materials and cell biology, it may eventually result in synthetic materials that contain the necessary signalling to run through development processes in tissue- and organ-specific differentiation and morphogenesis [20].

This would be ideal for topical application of the MSCs demonstrating significant accelerated wound closure and improved quality of cutaneous regeneration with an increased number of hair follicles and sebaceous glands when compared to intradermal injection strategies [18].

On the other hand based on the nature of wounds and healing approaches, several materials have been developed for use in wound dressings and bandages [21]. The crucial requirements of an ideal wound dressing includes: pain control, easy replacement, transparency to facilitate the healing follow up, absorption and prevention of body fluid losses, existing barrier against bacteria, oxygen ($O_2$) permeability, easy handling, and drug dosage control. Polysaccharides based hydrogels are good materials for wound dressings since they can adhere to the wound side protecting it from external milieu. They are transparent, so the clinical observation of wound site and healing process can be easily monitored; they also can be tailored into various shapes and sizes, they are permeable to $O_2$ and carbon dioxide ($CO_2$) but prevent the passage of microorganism [15].

The PVA is a water-soluble synthetic polymer and is prepared via the hydrolysis of poly(vinyl-acetate), in which the acetate groups are replaced by hydroxyls. In medical devices, PVA is being used as a biomaterial due to its biocompatibility, swelling properties, bio-adhesive characteristics, and for being non-toxic and non-carcinogenic [6, 22]. PVA may mimic the regulatory characteristics of natural ECMs and ECM-bound growth factors, both in

clinical applications and in basic biology studies [20].

The two patients referred to UPVET Hospital – ICBAS, University of Porto, Portugal, were previously treated with standard treatment protocols with no clinical improvement; and in both animals it was not possible to perform free grafts or rotational flaps. So, it was decided to use human MSCs (hMSCs) from Wharton's jelly therapy associated to the application of a PVA membrane. The results are presented and discussed here.

## Materials and Methods

### Poly(vynil-alcohol) hydrogel (PVA) membranes

Membranes of PVA (Aldrich, Mowiol 10-98) with a thickness of about 1 mm were prepared using a casting technique. A 15% (w/v) aqueous solution of PVA was used. The membranes were produced by a freezing/thawing process consisting in three cycles of freezer (-30ºC) / incubator (25ºC). The membranes were hydrated during 2 h before use.

### Cell Culture of human MSCs (hMSCs) from Wharton's jelly umbilical cord, viability and karyotype analysis

Human MSC from Wharton's jelly UC (hMSCs) were purchased from PromoCell GmbH (C-12971, lot-number: 8082606.7). Cryopreservated cells were cultured and maintained in a humidified atmosphere with 5% $CO_2$ at 37ºC. Mesenchymal Stem Cell Medium (PromoCell, C-28010) was replaced every 48 hours. At 90% confluence, cells were harvested with 0.25% trypsin with EDTA (Gibco) and passed into a new flask for further expansion. hMSCs at a concentration of 2500 cell/ml were cultured and after 24 hours cells exhibited 30-40% confluence. Mesenchymal Stem Cell Medium (PromoCell, C-28010) was replaced every 48 hours. The phenotype, the cell morphology, adherence rate and viability of the hMSCs was assessed by PromoCell by rigid quality control tests. Each cell lot was characterized by flow cytometry analysis for a comprehensive panel of markers [23]. The hMSCs established PromoCell cell line exhibited a mesenchymal-like shape with a flat and polygonal morphology in culture. During expansion the cells became long spindle-shaped and colonized the whole culturing surface (Figure 1). At 90% confluence, cells were harvested with 0.25% trypsin with EDTA (Gibco) and a suspension of 1000cells/µl was prepared in insulin syringes to facilitate the surgical procedure. The hMSCs infiltration was performed in a total volume of 100 µl per $cm^2$ of lesion area. Chromosome analysis on the hMSCs before *in vivo* application was carried out between passages 4 and 5, as previously described [23]. Chromosome analysis was performed by one scorer on 20 Giemsa-stained metaphases. Each cell was scored for chromosome number. Routine chromosome G-banding analysis was also carried out for determination of the karyotype. Intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) was measured in Fura-2-loaded cells by using dual wavelength spectrofluorometry as previously described [23, 24]. The measurements were performed on undifferentiated hMSCs cultured on PVA membrane discs after confluence was obtained, in order to correlate the hMSCs ability to expand and survival capacity in the presence of the biomaterial. The application of the hMSCs isolated from the umbilical cord Wharton's jelly in dogs is possible without inducing immunessupression, as previously referred.

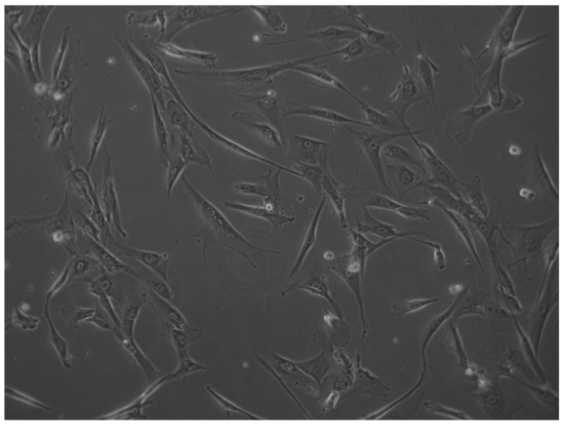

**Figure 1** - Undifferentiated human mesenchymal stem cells (hMSCs), from human umbilical cord Wharton's jelly, exhibiting a star-like shape with a flat morphology (Magnification: 100x).

### Physical examination and surgical procedure

The clinical cases concerning two dogs were referred for examination at UPVET Hospital – ICBAS, University of Porto, Portugal. In clinical case 1, a 2 years old, 32 kg neutered male mixed breed dog, presented a chemical burn injury with a 16-month history of clinical presentation and evolution and without any healing response to several and previous standard treatments. The lesion involved the left thoracic and abdominal wall and consisted of two main central and ulcerated areas, measuring 7.0x2.0 cm and 3x3 cm and surrounded by an exuberant fibrous scar (Figure 2A).

In the clinical case 2, a 5 years old, 34 kg spayed female mixed breed dog, presented a similar but smaller lesion, with a 24-month history of evolution and also refractory to the conventional treatments previously instituted. The ulcerated lesion was local-

ized in the sacro-iliac region and measured 6x5 cm, presenting peripheral fibrosis (Figure 3A). The cause of the injury was unknown. After a complete physical examination, the only abnormal finding in both animals was the deep and extensive skin ulcerated lesions affecting the epidermal and dermal layers. By the time of the surgery (pre-treatment biopsy), both animals were submitted to a skin biopsy performed with a scalpel blade number 23. Collected tissues were then fixed in 10% buffered formalin, paraffin-embedded, 3-µm thick cut and stained with haematoxylin and eosin (HE) for histological examination.

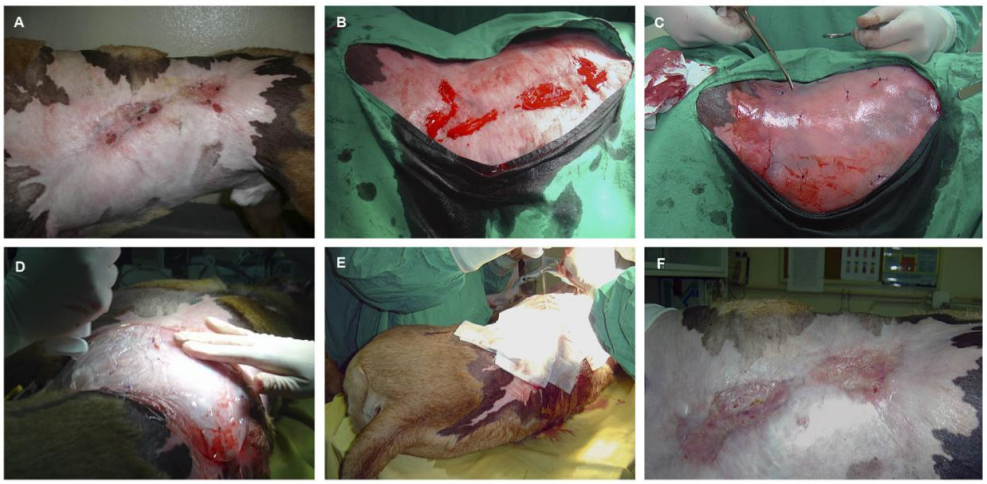


**Figure 2** – In clinical case 1, it can be observed an extensive chemical burn injury in the left thoracic wall with two central ulcerated areas presenting approximately 7cmx2cm and 3cmx3cm surrounded by an extensive fibrous scar **(A)**. Surgical biopsy and wound debridement **(B)**. PVA membrane application over the lesion area, after the hMSCs intradermal infiltration **(C)**. Moisture retentive bandage over the PVA membrane to prevent lesion dissecation **(D, E).** Complete cicatrization 2 months after human hMSCs local application **(F).**

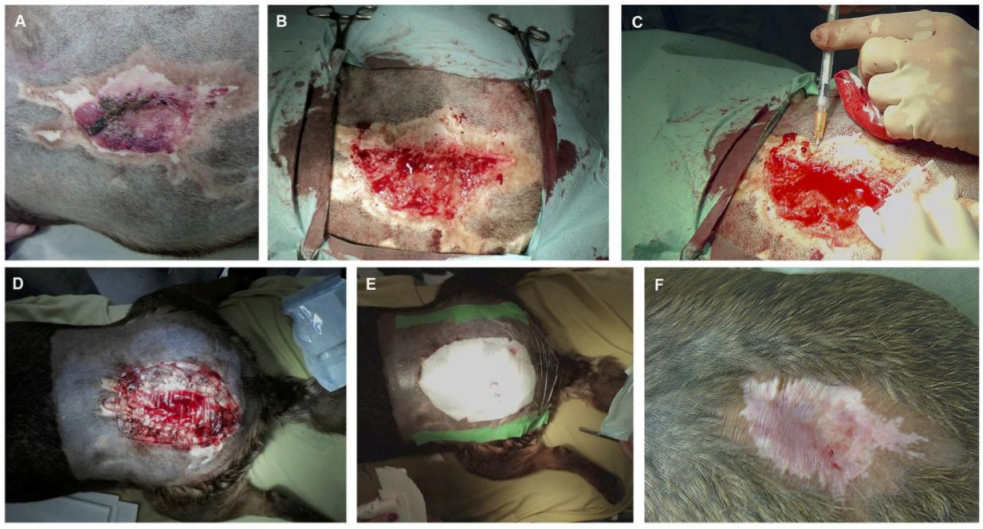


**Figure 3 –** In clinical case 2 the lesion was localized in the sacro-iliac region and measured approximately 6cmx5cm, with a central ulcerated and necrotic area presenting peripheral fibrosis. The lesion was of unknown cause (**A**). Surgical biopsy and wound debridement **(B).** Intradermal hMSCs application **(C)**. Moisture retentive bandage over the PVA membrane to prevent lesion dissecation **(D, E).** Complete cicatrization 2 months after human hMSCs local application **(F).**

Both animals were previously subjected to standard clinical treatments for inducing healing by second intention, like healing ointments, hydrogel bandages and sugar bandages. In clinical case 1, an improvement occurred (in the area correspondent to the fibrosis area) until cicatrization process stopped without complete closure of the wound. The wound remained without any sign of improvement for about 16 months.

For the hMSCs application, both animals were pre-medicated with morphine (0,5mg/kg) and diazepam (0,2mg/kg), induced with propofol (4mg/kg) and maintained with isoflorane 2%. The affected area was surgically prepared. The edges of the wound were debrided (Figure 2B and Figure 3B) and the undifferentiated hMSCs from Wharton's jelly of human umbilical cord, previously isolated and expanded *in vitro* were locally infiltrated at a concentration of 1000 cells/µl in culture medium (Figure 1, Figure 3C). The hMSCs infiltration was performed in a total volume of 100 µl per $cm^2$ of lesion area. Methylprednisolone was intravenously administered (Solu-medrol 125®, Pfizer, 1 mg / kg). Antibiotherapy was performed in both patients with Enrofloxacin 5 mg/kg once a day for 15 days. Finally, a sterile PVA membrane was applied to prevent desiccation of the wound (Figure 2C and Figure 3D), and a moisture-retentive dressing was applied over it and changed twice a day until day 3 after surgery (Figure 2D, Figure 2E, Figure 3E). Afterwards, the wound dressing was changed daily for the next 15 days. After this period, the PVA membranes were removed and non-adherent dressings were applied over the lesion area and daily changed during 10 more days. After complete cicatrization (2 months after the surgery) a second skin biopsy was performed to the clinical case 1, in attempt to evaluate the progress of the lesion. Unfortunately the second skin biopsy was not performed in clinical case 2, since the owners did not authorized it.

## Results

### hMSCs cell culture, viability and karyotype analysis

Undifferentiated hMSCs from umbilical cord Wharton's jelly exhibited a normal star-like shape with a flat morphology in culture (Figure 1). A total of 20 Giemsa-stained metaphases of these cells, were analysed for numerical aberrations. Sporadic, non-clonal aneuploidy was found in 1 cell (45 chromosomes). The other 19 metaphases had 46 chromosomes. The karyotype was determined in a completely analysed G-banding metaphase and no structural alterations were found. The karyotype analysis performed demonstrated once again, like in previous published experimental work [23], that this cell line has not neoplastic characteristics and is stable during the cell culture procedures in terms of number and structure of the somatic and sexual chromosomes. Results obtained from epifluorescence technique $[Ca^{2+}]_i$ measurements with the undifferentiated hMSCs corresponded to cells that did not begin the apoptosis process. The undifferentiated hMSCs cultured on PVA membranes reached confluence and exhibited a normal star-like shape with a flat morphology in culture. According to these results, it is reasonable to conclude that PVA membranes are a viable substrate for undifferentiated human MSCs adhesion, and expansion (Figure 1).

### Clinical follow-up and Histological Examination

There were no clinical complications in the post-operative period and after 2 months a complete epithelialisation was observed in both cases (Figure 2F and Figure 3F). The animals remained without any discomfort, pain or prurience throughout this period.

Prior to the hMSCs-based therapy with a PVA membrane procedure, in clinical case 1, microscopically, the affected skin presented an extensive and irregular epidermal hyperplasia, with various orthokeratotic hyperkeratosis and parakeratosis foci. Several and multifocal areas of ulceration covered by serocellular crusts containing numerous clusters of bacteria, associated with large amount of inflammatory infiltrate, consisting predominantly of neutrophils and fewer lymphocytes were also observed. The dermis displays images of exuberant fibrosis and dermal-epidermal dissociation. It was not possible to identify skin appendages (Figure 4A and Figure 4B).

In clinical case 2, focal epidermal ulceration was observed with the histological exam. The dermis was constituted in its almost entire length by dense connective tissue showing few nuclei and cells, with the presence of various hemorrhage and fibrinoid necrosis areas (Figure 5A and Figure 5B).

By the time of the second biopsy (2 months), in clinical case 1, the epidermal showed moderate orthokeratotic hyperplasia and presence of multifocal serocellular crusts. Areas of dermal fibrosis and absence of skin appendages were still prominent features however, in the superficial dermis, an exuberant granulation tissue was noted, rich in active fibroblasts and dilated neo-vessels coated by tall columnar endothelium. Moderate amount of diffuse mononuclear cell infiltration (macrophages, plasma cells and lymphocytes) was also present (Figure 4C and Figure 4D).

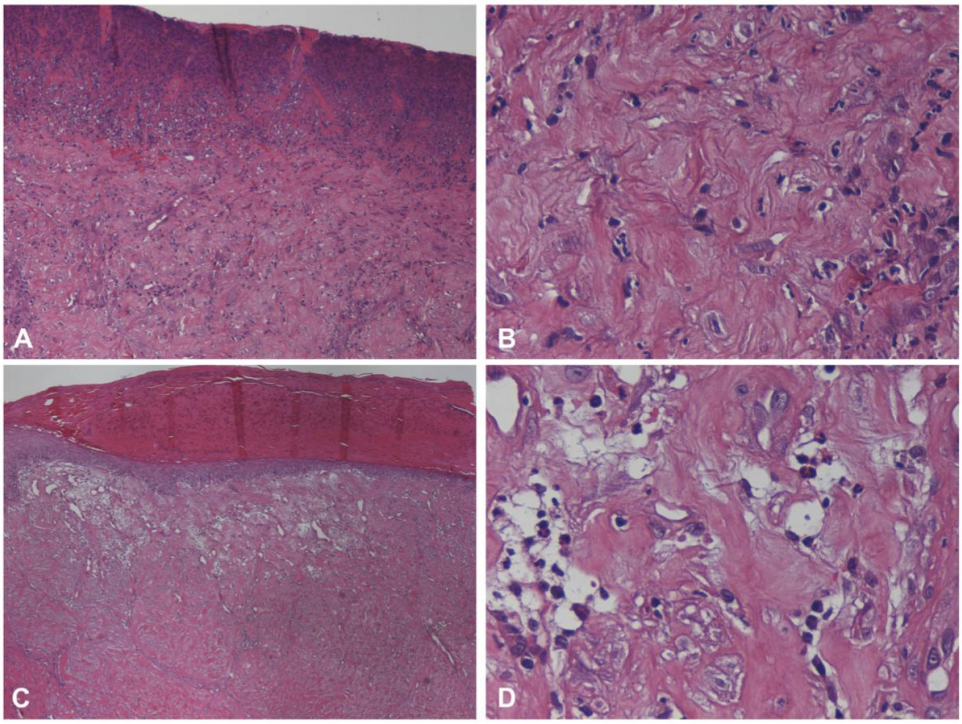


**Figure 4** - Histopathological findings of clinical case 1 skin biopsy. Before the scaffold application (pre-treatment biopsy), the affected skin presented severe and multifocal areas of ulceration (**A** and **B**), associated with large amount of inflammatory infiltrate consisting predominantly of neutrophils. The dermis displays images of exuberant fibrosis. It was not possible to identify skin appendages. This could be observed using HE and a magnification of 100x (**A**) and 400x (**B**). At day 60 after the hMSCs application, the epidermal showed signs of re-epithelization presenting orthokeratotic hyperplasia and multifocal serocellular crusts, HE, 40x (**C**). In the underlying dermis an exuberant granulation tissue was noted, with several fibroblasts and dilated neovessels containing neutrophils, HE, 400x (**D**).

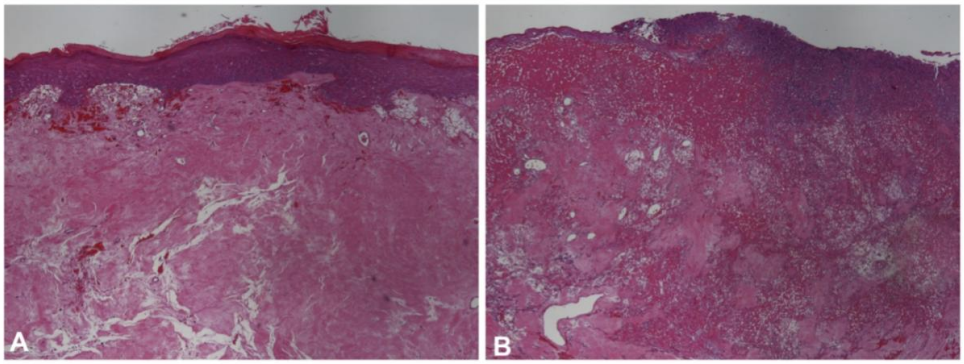


**Figure 5** - Histopathological findings of clinical case 2 skin biopsy. Before the scaffold application (pre-treatment biopsy), the dermis was constituted in its almost entire length by dense connective tissue showing few nuclei and cells, HE and magnification of 40x (**A**). Ulceration, hemorrhage and fibrinoid necrosis areas were also observed in the mid- to superficial dermis, HE and magnification of 40x (**B**).

## Discussion

Tissue engineering is a multidisciplinary research field that combines the ideologies of engineering and life sciences together to develop biological substitutes to restore, maintain or improve tissue function. Therefore there are emerging cellular therapies as an alternative for organ and tissue transplantation. MSCs have become one of the most interesting cells for tissue engineering since these cells present high plasticity, proliferative and differentiation capacity and immunosuppressive properties due to a decreased or even absence of HLA class II expression [25]. Currently the differentiation potential of MSCs in multi-lineage end-stage cells is already proven, and

their potential for treatment of cardiovascular [26], neurological [27], musculoskeletal [28], and cutaneous [1] diseases is now well established. MSCs can be obtained from many different tissues, including bone marrow, adipose tissue, skeletal muscle, umbilical cord matrix and blood, placental tissue, amniotic fluid, synovial membranes, dental pulp, foetal blood, liver and lung [29-34]. These cells are important for the Regenerative Medicine field because they have anti-inflammatory and immunomodulate properties by cytokines and growth factors production [35, 36]. As a matter of fact, the therapeutic effects of MSCs are due to their ability to repair damage tissue, to their capacity of modulating surrounding environment, and of activating endogenous progenitor cells [37, 38]. Also, several studies have demonstrated that MSCs, have a higher chromosomal stability and lower tendency to form tumours and teratomas, compared to other stem cells, for instance, embryonic stem cells (ESCs) [39-41].

The local application of hMSCs isolated from the umbilical cord Wharton's jelly together with a PVA membrane, was tested to promote wound healing in two dogs that were referred for clinical examination at UPVET Hospital, showing non-healing large skin lesions after standard treatments failure.

In these two clinical trials, the karyotype analysis of the hMSCs cell line derived from the Wharton's jelly locally applied, demonstrated that this cell line did not have neoplastic characteristics and was stable during the cell culture procedures in terms of number and structure of the somatic and sexual chromosomes. Also, the results obtained from $[Ca^{2+}]_i$ epifluorescence measurements performed with hMSCs cultured on PVA discs, confirmed that these cells that did not begin the apoptosis process and were viable cells. The undifferentiated hMSCs *in vitro* cultured on PVA membranes reached confluence and exhibited a normal star-like shape with a flat morphology in culture, which proved the citocompatibility of this biomaterial. According to these results, it is reasonable to conclude that PVA membranes are a viable substrate for undifferentiated hMSCs adhesion, and multiplication. The option to have chosen a physically, instead of the chemically, cross-linked PVA membranes aimed to avoid releasing of chemical compounds from a classic chemical cross-linking technique that could interfered with cell behaviour. As reported elsewhere [42], the freezing/thawing technique leads to anisotropic microstructure materials. The processing route chosen in this study produced PVA membranes with a structure, morphology and stability adequate to be used with cellular systems like the hMSCs from the umbilical cord Wharton's jelly.

The healing process is a complex and dynamic phenomenon which normally happens in a very orderly and efficient manner in order to restore the anatomical continuity and tissue function. The normal healing cascade begins with haemostasis and fibrin deposition which leads to an inflammatory cell cascade, characterized by neutrophils, macrophages and lymphocytes within the tissue. This is followed by attraction and proliferation of fibroblast and collagen deposition and finally remodelling by collagen cross-linking and scar maturation. If any part of this healing sequence is altered, pathologic responses can occur, leading to fibrosis and chronic ulcers [43]. In some pathological conditions the lesions stay trapped in a state of chronic inflammation characterized by abundant neutrophil infiltration, as described in clinical case 1 before the hMSCs and PVA application. The neutrophils release enzymes responsible for the destruction of the connective tissue matrix, for the inhibition of important healing factors and are associated with reactive oxygen species that cause cell damage. When tissues are injured, the fibroblasts synthesize collagen which is essential to repair the defect and restore the tissue anatomic structure and function [43]. However, if an excessive amount of collagen is deposited, the normal anatomical structure is lost, function is compromised and fibrosis occurs with reduced remodelling, similarly to that seen in clinical case 2 before the scaffold application. In both described clinical cases, the healing process would possible proceed if the inflammation was controlled and the wound bed was properly prepared.

Histological analysis over time of clinical case 1 indicated that hMSCs-treated wound showed more stable features in the epidermal layer compared with day 0, suggesting a more mature epithelium. A noteworthy change in the nature of the inflammatory infiltrate was recorded: neutrophils were gradually replaced by mononuclear cells associated with granulation tissue formation, suggesting that the classical healing sequence was overtaken. It was also observed that, by the time of the second biopsy, the treated wound was involved by a denser capillary network when compared with the biopsy analysis performed prior to the scaffold application, suggesting that the treatment applied promoted angiogenesis in the dermis. These results suggest that in clinical case 1, the hMSCs infiltrated in the wound accelerated epidermal and dermal renewal. Despite the absence of the second biopsy to confirm the histopathological evolution of the lesion, the clinical course of the case 2 is clearly remarkably since, as you can see in figure 3, panel F, the rate of wound closure and re-epithelialization was accelerated, the quality and strength of the regenerated tissue was improved and the visual appearance of the lesion was minimize. All

these evidences, taken together, suggest that the application of hMSCs could accelerate wound healing, promoting angiogenesis and providing the cells and cellular factors needed to create the optimal wound-healing microenvironment in canine chronic wounds.

Previous studies showed that MSCs exhibit a considerable and important number of trophic functions to enhance tissue regeneration (for review see the published work of Jackson et al., 2012 [44]), such as modulating the inflammatory response (by regulating the function of the leukocytes present in the lesion) and promoting angiogenesis. Once the MSCs enter the inflammatory environment due to chemotaxis, their immunomodulatory phenotype is activated by interferon-γ (IFNγ), tumour necrosis factor-α (TNFα) and interleukin-1β (IL-1β) [45]. It is also well documented the ability of MSCs to regulate T-cell recruitment, proliferation and activity [45] and their capability of suppressing the proliferation of B cells [46] and natural killer cells [47], thereby improving the acute immune response to injury [48]. By attenuating the function of these cell types, the MSCs would likely reduce the pro-fibrotic responses that can occur coincident with prolonged inflammation during wound healing [49]. Moreover, some studies reported that engrafted MSCs in the wound improved angiogenesis by exerting a paracrine effect which increases levels of angiogenic factors such as angiopoietin-1 and vascular endothelial growth factor (VEGF) [50].

In both clinical cases described, both patients were not good candidates to do rotational flaps or free grafts since the extension and location of the lesions did not allow obtaining healthy skin to transpose and the free grafts had very small chance to be successful since their irrigation would be very compromised by the skin architecture of the surrounding tissues. The results of our study reinforced the efficacy of therapy with hMSCs on enhancing wound healing in dogs by promoting epidermal and dermal regeneration and angiogenesis. However, further studies are needed in order to investigate if its administration may thus represent a novel therapeutic approach and to clinically explore further improvements in canine cutaneous wound therapy.

It was concluded that, in both clinical cases the association of hMSCs from Wharton's jelly of human umbilical cord and PVA membrane did not present any negative effect and even improved the wound healing, in two clinical cases where the standard treatments failed. This cellular therapy associated to a PVA membrane should be further investigated in order to be applied in the future in veterinary clinical practice as in human medicine.

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## Competing Interests

The authors have declared that no competing interest exists.

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# Capítulo IV – Discussão, conclusões e perspetivas futuras

## Capítulo IV – Discussão, conclusões e perspetivas futuras

As lesões de nervo periférico ocorrem frequentemente no Homem com consequências dramáticas (Whitlock, Tuffaha et al. 2009, Schmitte, Tipold et al. 2010). Nas outras espécies, em particular em animais de companhia, as lesões de nervo periférico resultam de fraturas ósseas, com compressão ou seccionamento nervoso ou avulsões traumáticas dos plexos nervosos, especialmente o plexo braquial.

Apesar do constante desenvolvimento de diferentes técnicas de microcirurgia para reparação do nervo periférico, a recuperação é frequentemente insatisfatória, especialmente em lesões de neurotmese com perda de massa nervosa, resultando um afastamento dos topos nervosos. Nestes casos, a restrição à utilização de técnicas de microcirurgia cujos resultados estão já maximizados, é ainda limitante, para a recuperação funcional, pelo que nos últimos anos, quer do ponto de vista da aplicação clínica quer do ponto de vista da investigação, este problema tem sido abordado de uma forma multidisciplinar, envolvendo áreas como a medicina humana, medicina veterinária, engenharia, fisiologia, biologia, bioquímica, fisioterapia ou farmacologia (Geuna, Gnavi et al. 2013).

A medicina regenerativa surge assim cada vez mais como centro de uma multiplicidade de ramos de investigação (Geuna, Gnavi et al. 2013). Os trabalhos desenvolvidos no âmbito desta tese de Doutoramento procuraram dar algum contributo para a melhoria da compreensão, mas fundamentalmente na procura de novas soluções que melhorem a regeneração do nervo periférico. Assim procurou-se desenvolver membranas e tubos-guia para serem aplicados em lesões nervosas e associar estes biomateriais a sistemas celulares que potenciem a regeneração celular.

Este trabalho focou-se no desenvolvimento de tubos-guia electrocondutores cujo material base é o poli (álcool vinílico) (PVA), e a sua condutividade eléctrica foi melhorada através da incorporação de diversos agentes. Numa segunda fase, estes tubos foram aplicados em lesões sub-criticas (lesões de axonotmese). Na terceira fase dos trabalhos experimentais testou-se o desempenho deste biomaterial em lesões críticas (lesões de

neurotmese) assim como a associação destes biomateriais a terapias celulares (aplicação de MSCs isoladas da geleia de Wharton) para verificar o seu impacto na regeneração de nervo periférico.

A escolha do PVA prendeu-se com o facto de ser um material biodegradável, suave, permeável, capaz de formar interfaces com tecidos vivos (Kobayashi and Hyu 2010), biocompatível (Alexandre, Ribeiro et al. 2014) e apresenta ainda a possibilidade de poder ser combinado com diversas moléculas que aumentam a sua condutividade, tendo sido testadas nos nossos trabalhos *in vitro,* o polipirrol, nanotubos de carbono, nitrato de prata e cloreto de magnésio em diferentes concentrações. Estas características levaram-nos a testar este biomaterial, apesar de ser um material não absorvível.

Dados os resultados de condutividade obtidos nos testes *in vitro*, foram selecionados para implantação nos modelos animais de axonotmese e de neurotmese, o PVA sem qualquer tipo de combinação molecular, o PVA associado ao polipirrol (à concentração de 0,05%) e o PVA associado a nanotubos de carbono (*Multiwall carbon nanotubes – MWCNTs* - à concentração de 0,05%).

O início do estudo consistiu na produção de tubos-guia e membranas de PVA, cujas características físicas tornassem possível a sua aplicação em nervo periférico, especialmente em termos de resistência e suturabilidade.

A biocompatibilidade do biomaterial tinha já sido testada em trabalhos prévios realizados pelo nosso grupo de investigação (Alexandre, Ribeiro et al. 2014) onde verificamos que o biomaterial é apenas ligeiramente irritante para os tecidos adjacentes de acordo com a Norma ISO 10993-6 (Anexo E).

A terceira fase do estudo passou pelo estudo *in vitro* do PVA e as suas associações a diferentes moléculas eletrocondutoras, onde se concluiu que a associação ao polipirrol (PPy) e com CNTs aumentou significativamente a condutibilidade elétrica dos tubos-guia em todas as concentrações testadas. Este estudo também demonstrou o comportamento hidrofílico de todos os tubos, embora o PVA associado com o polipirrol fosse ligeiramente mais hidrofóbico que os outros tubos testados. Os tubos-guia de PVA associados aos CNTs mostraram uma topografia mais rugosa relativamente aos outros, o que é uma característica benéfica para a associação de sistemas celulares, por aumentar a adesão e expansão celular. Esta associação tornou também estes

tubos-guia menos rígidos. Ainda de acordo com os resultados obtidos utilizando a técnica de epifluorescência, concluiu-se que os três biomateriais escolhidos para testagem *in vivo* PVA, PVA com CNTs (0,05%) e PVA com PPy (0,05%) são substratos viáveis para a expansão e sobrevivência de MSCs isoladas da geleia de Wharton do cordão umbilical, pois o processo de apoptose destas células não foi iniciado na presença de nenhum dos três biomateriais testados.

O passo seguinte foi a testagem *in vivo* dos biomateriais escolhidos e sua aplicação em lesões axonotmese de nervo periférico, sendo utilizado o rato como modelo animal experimental, inicialmente descrito por Varejão e colaboradores (Varejão ASP 2004), tendo sido realizado o seguimento funcional durante um período de 12 semanas pós-cirúrgicas, recorrendo-se à medição do WRL, EPT, SFI e SSI, à avaliação cinemática no final das 12 semanas imediatamente antes do abate dos animais para realização de histomorfometria do nervo e do músculo *tibialis anterior* assim como análise histológica de órgãos internos após eutanásia dos animais.

A histologia dos órgãos internos é no caso da utilização de CNTs importante, pois revela a ausência de resíduos resultantes do biomaterial nos órgãos internos quando utilizados à concentração de 0,05%, provando-se não ser danosas para o organismo.

Nos trabalhos experimentais em que realizamos lesões de axonotmese, a histomorfologia do músculo *tibialis anterior* demonstrou que nos grupos em que foram aplicadas membranas de *PVA-PPY* e *PVA-CNTs* houve um aumento médio de 9% e 19% nos tamanhos da área das fibras musculares e de 5% e 10% do diâmetro mínimo de Feret, respetivamente, em comparação com o grupo controlo. Este facto é de extrema importância, pois nas lesões de nervo periférico, um dos fatores que condiciona de sobremaneira a recuperação funcional é a atrofia muscular que rapidamente se instala em lesões de motoneurónio inferior.

A histomorfometria do nervo periférico regenerado permite comparar o número de fibras mielinizadas, o diâmetro axonal, a espessura de mielina e o cálculo do rácio-g permite apreciar a maturação das fibras mielinizadas. O estudo destas variáveis permitiu verificar que o número de fibras mielinizadas em todos os grupos não apresentava diferenças estatisticamente significativas,

no entanto o grupo *PVA-CNTs* apresentava uma maior espessura da camada de mielina e um rácio-g inferior, o que demonstra uma melhor maturação das fibras mielinizadas, facto que é também suportado pelos melhores resultados deste grupo nos estudos da cinemática.

No estudo de axonotmese ficou então demonstrado o efeito positivo da utilização de membranas com maior condutibilidade elétrica (*PVA-PPy*, mas principalmente *PVA-CNTs*) que pode ser explicado pelo fenómeno de *sprouting* que acelerando e melhorando o processo de recuperação, permite além da recuperação histológica das fibras nervosas, uma menor diminuição da atrofia muscular, fundamental para a recuperação funcional.

Este estudo demonstrou que a utilização de tubos-guia eletrocondutores é uma via de investigação promissora e que a utilização de CNTs a 0,05% associado ao PVA é biocompatível. Tais conclusões conduziram a que fosse escolhido o *PVA-CNTs* como biomaterial de eleição para associar a MSCs no estudo subsequente em lesões de neurotmese.

No estudo em lesões de neurotmese foram aplicadas as mesmas metodologias que no estudo de lesões de axonotmese, sendo o tempo de seguimento pós-cirúrgico de 20 semanas. Foram estudadas grupos em que se aplicou tubos-guia de *PVA, PVA-PPy, PVA-CNTs, PVA-CNTs-MSCs* e grupos controlo de sutura topo-a-topo e autoenxerto. Nestes grupos, não houve diferenças estatisticamente significativas nos testes funcionais EPT, WRL e o SFI não foi possível calcular devido à autotomia em todos os grupos. No entanto nos grupos *PVA-PPy* e *PVA-CNTs-MSCs* apresentaram melhores resultados de SSI relativamente ao grupo de autoenxerto, apesar de não se verificar em nenhum dos grupos melhoria da velocidade de recuperação ao nível dos testes funcionais.

Na análise cinemática da articulação do tornozelo não houve diferenças estatisticamente significativas entre grupos nos tempos IC, OT e HR, mas no tempo TO, o grupo *PVA-CNTs-MSCs* apresentava ângulos menos agudos que os outros grupos experimentais o que pode sugerir uma melhoria na função muscular dos músculos implicados na articulação do tornozelo, envolvidos na fase de saída da parte distal do pé.

No estudo morfométrico do músculo verificou-se que os grupos *PVA-CNTs* e *PVA-PPy* em termos de tamanho médio das fibras musculares e de diâmetro

mínimo de Feret, apresentaram valores superiores de mais 42% e 21% e de 25% e 13% respetivamente, quando comparados com os valores do grupo com autoenxerto, embora ainda menores que o músculo normal. Os grupos de *PVA* e *PVA-CNTs-MSCs* não mostraram benefícios em comparação com o autoenxerto na avaliação da atrofia muscular, sendo que este último grupo além da atrofia muscular foi detetada considerável necrose associada ao atraso da regeneração muscular, resultado não correlacionável com os resultados de SSI e da análise cinemática.

A histomorfometria do nervo demonstrou regeneração das fibras nervosas em todos os grupos com densidades de fibras nervosas semelhantes em todos os grupos, embora quando comparados com nervo sem lesão todos os grupos apresentaram axónios menores, bainhas de mielina mais finas e microfasciculação. O grupo *PVA-PPy* apresentou diâmetro axonal maior que todos os outros grupos e o grupo *PVA-CNTs-CNTs* apresentou maior espessura da bainha de mielina. Estes resultados podem ser explicados no caso do *PVA-PPy* pelo facto de o tubo-guia ser eletrocondutor aumentar a orientação das fibras ao longo de espaço entre os topos nervosos e no caso do *PVA-CNTs-MSCs*, o aumento da bainha de mielina (revelado pelos valores M, M/d e D/d na estereologia, semelhantes aos grupos controlo de sutura topo-a-topo e de autoenxerto) pode ser explicado pelo efeito sinergístico que as células estaminais têm e pelo fatores neurotróficos que produzem, aumentando os efeitos nas células de Schwann e o processo de mielinização (Lu, Jones et al. 2005, Caplan and Dennis 2006, Zhang, Zhang et al. 2009, Ankrum and Karp 2010, Bonfield, Nolan Koloze et al. 2010, F Azari, Mathias et al. 2010, Joyce, Annett et al. 2010, Meyerrose, Olson et al. 2010, Shen, Duan et al. 2010, Cheng, Duan et al. 2011, Ladak, Olson et al. 2011, Liu, Cheng et al. 2011, Lopatina, Kalinina et al. 2011, Gartner, Pereira et al. 2012, Gärtner, Pereira et al. 2013).

A recuperação funcional nas lesões de nervo periférico é, apesar dos diversos avanços técnicos e tecnológicos, ainda uma área de investigação com grande margem de progressão em diversos campos de investigação, pois apesar das melhorias obtidas, o resultado final é ainda insatisfatório.

A melhoria da recuperação funcional passará pela multidisciplinaridade e combinação de forma sinergística dos efeitos benéficos de diversas terapêuticas.

O desenvolvimento de biomateriais eletrocondutores na produção de tubos-guia parece ser uma janela no futuro do desenvolvimento da regeneração do nervo periférico (Al-Majed AA 2000a, Al-Majed AA 2000b, Brushart TM 2002, Brushart T M 2005, Chronakis, Grapenson et al. 2006, Willand, Nguyen et al. 2015). Associado a este tipo de biomateriais aparece a estimulação elétrica dos nervos lesados, que em alguns estudos foi demonstrado que a aplicação de estímulos elétricos por breves períodos foi associada a um menor tempo de regeneração (Al-Majed AA 2000a, Al-Majed AA 2000b, Brushart TM 2002, Brushart T M 2005, Chronakis, Grapenson et al. 2006, Willand, Nguyen et al. 2015). Este parece ser um ponto fulcral na regeneração do nervo periférico. A diminuição do tempo de regeneração é de extrema importância, pois diminui na fase inicial da regeneração axonal, os fenómenos de degenerescência e a aceleração da regeneração diminui também a atrofia muscular, sendo este último ponto determinante para a recuperação funcional.

No mesmo sentido, quer o exercício voluntário quer a fisioterapia, parecem estimular o crescimento axonal (Sabatier, Redmon et al. 2008, Bischoff, Grosheva et al. 2009, Deumens, Bozkurt et al. 2010), embora o mecanismo que medeia este efeito esteja ainda mal esclarecido.

A utilização de células estaminais de diversas origens assim como de células diferenciadas a partir de células estaminais são uma arma terapêutica muito promissora dada a sua capacidade de diferenciação, mas sobretudo pelos efeitos parácrinos que apresentam, e que estão ainda longe de ser maximizados (Alhadlaq and Mao 2004, Kingham, Kalbermatten et al. 2007, Cho, Jang et al. 2010, Mantovani, Mahay et al. 2010). Tem também de ser realçada a possibilidade de aplicação destas terapêuticas noutros tecidos como os que foram realizados em simultâneo, durante os trabalhos que conduziram a este Doutoramento em lesões crónicas de pele, onde foram aplicadas MSCs e recobertas com uma membrana de PVA, com resultados bastante promissores.

O futuro da investigação passará obrigatoriamente pelo desenvolvimento dos efeitos da aplicação de MSCs, pelo desenvolvimento de novos biomateriais absorvíveis, eletrocondutores como as membranas piezoelétricas que se

encontram já em fase de testagem *in vivo*, pelo nosso grupo de investigação e pela terapia genética. O nosso grupo de trabalho encontra-se neste momento a testar membranas piezoelétricas em ratos, a isolar e multiplicar *in vitro* diversas linhagens de MSCs de diferentes espécies (como por exemplo, canideos, felídeos, ovinos) e de diferentes origens (cordão umbilical, polpa dentária, entre outras), sendo estas áreas de investigação extremamente promissoras, no sentido de aplicação clínica autóloga e alogénica.

Os estudos efetuados no âmbito deste Doutoramento visaram estudar fundamentalmente os efeitos de membranas e tubos-guia com capacidade eletrocondutora e os seus efeitos na regeneração de nervo periférico em lesões de axonotmese e de neurotmese e visaram estudar a sua biocompatibilidade e citocompatibilidade, com os tecidos onde seriam aplicadas e com as células estaminais que foram aplicadas concomitantemente. Pretendeu-se ainda estudar os efeitos destas células e os efeitos sinergísticos obtidos entre estas células e os biomateriais aplicados. Considera-se que estes estudos abrem perspetivas promissoras nestas áreas de investigação.

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